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Functional characterization of the CCR4-NOT transcriptional regulatory complex

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FUNCTIONAL CHARACTERIZATION OF THE CCR4-NOT TRANSCRIPTIONAL REGULATORY COMPLEX

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DISSERTATION

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DEDICATION

To my parents and teachers for inculcating and encouraging the curiosity and passion for science.

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I am grateful for the love, support and immense patience of my wife Monika, all which were integral to the success of this endeavor. I am thankful for the unconditional love of my parents and my brother, Vivek. I also thank my parents for providing the direction and their guidance throughout my life. I am grateful to Dr.Clyde Denis for the opportunity and guidance to carry out this research and also for being much more than a thesis advisor. I am thankful to the members of the Denis lab, past and present, for their help, support and advice. I would like to thank Queta Boese for her help and advice in dealing with several situations encountered as a Graduate student and also for many stimulating and fruitful discussions.

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ABSTRACT

The CCR4-NOT transcriptional regulatory complex affects expression of a number of genes both positively and negatively. This study demonstrates that the CCR4-NOT complex functionally and physically interacts with TBP and TAFs. Firstly, mutations in CCR4, NOT4, and NOT5 suppressed the his4-912 delta insertion by a mechanism similar to that observed for the defective TBP allele spt15-122. This mechanism appeared to involve stabilization of TBP binding to a specific non-consensus TATA sequence, CATAAA, in the his4-912 delta element. Secondly, using modified HIS3 promoter derivatives containing specific mutations within the TATA sequence, it was found that the NOT proteins were general repressors that disrupt TBP function irrespective of the DNA sequence. Thirdly, increasing the dosage of NOT1 specifically inhibited the ability of spt15-122 to suppress the his4-912 delta insertion but did not affect the Spt-phenotype of either spt3 or spt10 at this locus. Fourthly, spt3, spt8 and spt15-21 alleles (all involved in affecting interaction of SPT3 with TBP) suppressed ccr4 and caf1 defects. Also, a number of genetic interactions were observed between CCR4-NOT complex components and TBP and TAFs

In addition, NOT2 and NOT5 were found to physically interact with TBP, multiple TAFs, and SPT3. Moreover, NOT2 and NOT5 were found to associate with components from purified SAGA and TFIID complexes, respectively. These genetic and physical interactions indicate that one role of the CCR4-NOT complex is to inhibit functional TBP-DNA interactions, perhaps by interacting with and modulating the function of factors which associate with TBP such as the components of TFIID and SAGA.

INTRODUCTION

Controlling the rate of RNA synthesis within the cell must be highly coordinated. Three different polymerases are used to transcribe rRNA, mRNA and small RNAs. Since the TATA Binding Protein (TBP) is the only factor that is shared among all three types of polymerases that transcribe these genes, it is a likely target for regulation of transcription by all three polymerases (Lee and Young 1998). In addition, the cell needs regulatory systems to modulate gene specific and global transcription in response to environmental stimuli. Since TBP binding to the TATA element is a rate limiting step for transcription of RNA polymerase II (Pol II) genes, several mechanisms for regulating transcription have evolved that target this process.

A number of factors have been identified that physically associate with TBP and stabilize its binding to DNA and are required for optimal TBP function at a subset of promoters. These factors include TAFs, TFIIA SPT3 and SPT8 (Hampsey 1998).

TAFs (TBP Associated Factors)

TBP is known to exist in at least four different complexes SL1, TFIID, TFIIIB and SNAPc that function in the expression of Pol I specific, Pol II specific, Pol III specific and small RNA genes respectively (Lee and Young 1998). TFIID which is involved in Pol II transcription consists of TBP and thirteen different TAFs (S.Hahn 1998). In vitro, TAFs were found to be dispensable for basal transcription but were essential for activated transcription (Pugh et al 1992). Therefore, TAFs were considered to be mediators of activated transcription. Consistent with this TAFs have been shown to

contact the activation domains of several different activators, bind other general transcription factors, and interact with promoter elements (Burke et al 1997; Chen et al 1994; Goodrich et al 1993; Verrijzer et al 1996). Thus TAFs were proposed to function as co-activators that convey information from the activators to the core transcription machinery. Recent studies done in yeast indicate that although TAFs are essential for viability, they are not required for activated transcription of several genes in vivo (Moqtaderi et al 1996; Walker et al 1996). It has also been reported that certain TAFs such as TAF130 bind DNA and function as promoter selectivity factors (Shen et al 1997). A subset of TAFs including $TAF_{II}130$, $TAF_{II}19$, $TAF_{II}47$, and $TAF_{II}40$ have been shown to be required for optimal expression from promoters containing non-consensus TATA elements, such as the T_C element of the HIS3 promoter and the TRP3 promoter (Moqtaderi et al 1996, 1998).

Another subset of TAFs consisting of the proteins that contain the "histone fold" motif, including $TAF_{II}61$, $TAF_{II}60$ and $TAF_{II}17$ have been reported to be required for the expression of a large subset in the yeast genome and are also important for activated expression for activated transcription of several inducible genes (Holstege et al 1998; Micheals et al 1998). These histone fold containing TAFs are components of multiple transcription regulatory complexes including TFIID and SAGA. The mechanisms of action of these TAFs remains unclear.

SAGA (Spt Ada Gcn5 Acetyl transferase)

SAGA is a large co-activator complex. It consists of at least three known classes of proteins involved in transcriptional regulation. Proteins from each class are known to

interact with TBP and are thought to stabilize its binding to DNA (Sterner et al 1999). The three classes of proteins include the ADA proteins, the SPT proteins and a subset of TAFs. The ADA proteins consist of ADA2, ADA3, ADA5/ SPT20 and GCN5 which is an histone acetyl transferase that acetylates specific lysine residues in the amino-terminal tails of the H3 and H4 histones (Berger et al 1992; Horiuchi et al 1995; Marcus et al 1996; Brownell et al 1996). This activity of GCN5 is essential for its co-activator function and is thought to alter nucleosome structure so as to allow increased access of transcription factors to DNA. The ADA proteins have also been shown to function as adaptors, bridging interactions between activation domains and components of the core transcription machinery (Barlev et al 1995). The TAFs that are part of SAGA include $TAF_{II}90$, $TAF_{II}23$ and the previously mentioned histone-like TAFs (Grant et al 1998). The role of these TAFs in the SAGA complex remains unclear.

The SPT proteins that are part of SAGA include SPT3, SPT7, SPT8 and SPT20 (Grant et al 1997). Mutations in these SPT genes have effects similar to specific mutations in TBP, on transcription from certain promoters (Winston et al 1984; Winston 1992). Isolation of allele specific suppressor mutations in SPT3 and TBP suggested a physical interaction between these two proteins which was subsequently confirmed by biochemical studies (Eisenmann et al 1994). A recent report indicates that SPT8 is essential for interaction between the SAGA complex and TBP (Sterner et al 1999). It is thought that SPT3 and SPT8 function to stabilize TBP binding to DNA and are important for TBP function at certain promoters (Eisenmann 1994; Madison 1997).

TFIIA

TFIIA is another factor that functions to stabilize TBP binding to DNA. TFIIA was initially identified as a general transcription factor since it was essential for transcription in in vitro systems using partially purified preparations of transcription factors (Reinberg et al 1987). Recent reports indicate that TFIIA is dispensable for basal transcription but is required for TFIID dependent activated transcription (Ozer et al 1994; Sun et al 1994). TFIIA has been shown to bind TBP-DNA complexes and form a stable ternary complex (Buratowski et al 1992; Imbalzano et al 1994). TFIIA has also been shown to interact with specific activators and certain TAFs (Sun et al 1994; Yokimori et al 1993). Recent evidence indicates TFIIA has an "Anti-Repression" function (Ma et al 1996) wherein it displaces TBP specific repressors like MOT1 and the NC2 complex from TBP or TFIID.

TBP specific repressors also play important roles in regulating global transcription as well as gene-specific transcription in response to environmental stimuli. This class of repressors includes MOT1 and the NC2 complex. Deletion of any one of these genes is lethal, emphasizing the importance of repressing TBP function.

MOT1

MOT1 functions to dislodge TBP from DNA in an ATP dependent manner (Auble et al 1993, 1994). MOT1 has ATPase activity that is essential for this function. Recent evidence suggests that MOT1 redistributes TBP between functional and non-functional TATA elements (Auble 1999). The TBP dislodging activity of MOT1 can be counteracted by TFIIA and mutations in MOT1 are synthetic lethal with TFIIA or SPT3

mutations (Madison et al 1994). This suggests that defect in MOT1 function reduces recruitment of TBP to functional TATA elements and TFIIA and SPT3 can partially offset this by stabilizing TBP binding to functional TATA elements.

NC2 complex

The NC2 complex has been shown to bind TBP and prevent access of TFIIA and TFIIB (Goppelt et al 1996a, 1996b). The NC2 complex is a heterodimer consisting of NC2 alpha and NC2 beta (Goppelt 1996b). Mutations in NC2 components allow the expression of several genes under repressing conditions (Prelich 1997). Overexpressing NC2 causes toxicity that can be alleviated by overexpressing TBP (Kim et al 1997). These data support the role of NC2 in modulating TBP availability at certain promoters. A role for NC2 in regulating global transcription is suggested by the fact that mutations in NC2 components can suppress the global transcription defects caused by an srb4 temperature sensitive mutation (Gadbois et al 1997).

SRB4 is an essential component of the RNA polymerase $_{II}$ holoenzyme. Mutations in SRB4 cause a rapid and dramatic drop in polyA transcript levels, when shifted to non-permissive temperature (Thompson et al 1995). Mutations in NC2 were found to suppress this effect. Other mutations that were isolated as suppressors of srb4-ts and therefore, likely to have global roles in transcription regulation include, not1, not3 and caf1 (Lee et al 1998) These genes encode components of the CCR4-NOT transcription regulatory complex.

CCR4-NOT complex

CCR4 was originally isolated as a factor required for optimal expression of the ADH2 gene (Denis 1984). Since then it has been shown to affect the expression of several genes including other genes involved in non-fermentative metabolism, genes involved in maintenance of cell-wall integrity, methionine biosynthesis and genes involved in DNA repair (Malvar and Denis 1992; Schild 1995; Mckenzie et al 1993; Tabtiang and Herskowitz 1998; Collart and Struhl 1993; Lee et al 1998; Liu et al 1998; Benson et al 1998). CCR4 exists as part of a large protein complex. Several components of this complex have been identified, including CAF1, DBF2 and recently the NOT proteins, NOT1 through NOT5.

The NOT genes were isolated as repressors of "T_C" initiated HIS3 transcription (Collart et al 1993; Collart et al 1994). As seen in Figure 2, the HIS3 promoter contains two important elements, "T_R" which consists of the canonical TATA sequence and is known to be required for activated transcription, and "T_C" which confers constitutive expression and consists of a long, ill-defined sequence containing several non-consensus TATA sequences. The NOT genes were isolated as mutations that cause increased transcription from the T_C element, in the presence of a weak activator (Collart et al 1993; Collart et al 1994). However it was not known whether this effect was specific to the T_C element or if it affected TBP binding to DNA. Further, the not mutations did not display any significant effects on HIS3 transcription in the presence of a strong activator GCN4 (Collart et al 1993; Collart et al 1994). GCN4 is a DNA binding acidic activator that activates transcription of several genes involved in amino acid biosynthesis including

HIS3 (Chen et al 1989). As described previously (Liu et al 1998, Appendix 1), the CCR4-NOT complex affects gene expression both positively and negatively. The CCR4-NOT complex consists of CCR4, CAF1, NOT1, NOT2, NOT3, NOT4, NOT5 and other proteins that are currently being characterized in the Denis lab.

The CCR4 protein contains a cluster of five Leucine Rich Repeat (LRR) motifs which are known to be involved in mediating protein -protein interactions (Suzuki et al 1990; Lee et al 1990; Draper et al 1994).CAF1 was isolated in a genetic screen for proteins that interact with CCR4 protein (Draper et al 1995). Both CCR4 and CAF1 when mutated display very similar phenotypes such as caffeine sensitivity, inability to grow on non-fermentative carbon sources at 37*C and reduced expression of ADH2 under derepressed conditions (Draper et al 1995; Liu et al 1998). CAF1 was also independently isolated as a negative regulator of PGK expression in stationary phase (Sakai et al 1992). NOT1, NOT2 and NOT4 have been previously isolated as mutations that allow the expression of pheromone inducible genes in

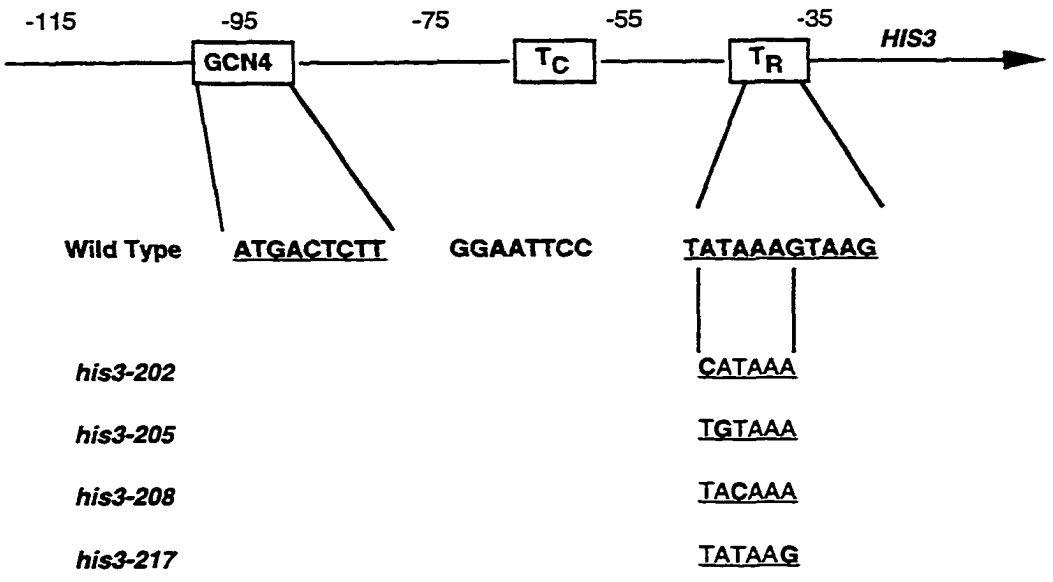


Figure 1: Schematic diagram of the normal HIS3 promoter and its derivatives.

The structure of the HIS3 promoter as described in Harbury et al 1989 is shown. Top : The GCN4 binding site, and T_C and T_R TATA elements are indicated with respect to the +1 mRNA initiation site.

Bottom : wild type - modified HIS3 promoter containing a single TATA element (Harbury and Struhl 1989) wherein the T_C and T_R elements have been replaced by the indicated sequences.his3-202, -205, -208, and -217 - same as wild type except the TATAAA element has been altered as indicated in bold.

the absence of pheromone stimulation (Cade et al 1994; Barros Lopes 1990). As a result, transcription of FUS1 which is normally repressed in the absence of pheromone stimulation is increased in strains mutated for these not genes (Cade et al 1994).

NOT1 is a large protein consisting of 2108 amino acids and is the only protein within the CCR4-NOT complex that is essential for viability (Collart 1994). The LRR region of CCR4 is necessary and sufficient for interaction between NOT1 and CCR4 (Draper et al 1994; Liu et al 1998). NOT1 migrates as a doublet of 195 kD and 185 kD. It has been shown that the smaller species is an N-terminal truncation (Liu et al 1998). Both species have been shown to interact with CCR4 (Draper et al; Liu et al 1998).

NOT2 is a small protein of 192 amino acids. Among the NOT genes, deletion of not2 has the most dramatic effects on ADH2 expression (Liu et al 1998, Appendix). The close functional and most likely physical interaction between NOT1 and NOT2 is indicated by the fact that the not1-2 mutation is specifically suppressed by the not2-4 mutation (Collart 1994) . A recent report indicates that the evolutionarily conserved C-terminal region of NOT2 is involved in functions similar to and overlapping with CCR4, while the N-terminus of NOT2 is involved in a distinct function (Benson 1999).

NOT4 encodes a protein of 587 amino-acids and contains a putative RING finger motif. RING finger motifs have previously been shown to be involved in mediating protein-protein interactions (Saurin et al 1996; Meza et al 1999). As stated earlier, NOT4 was isolated as a mutation that causes constitutive expression of the FUS1 gene, in the absence of pheromone stimulation. This phenotype is also conferred by a dominant negative allele of NOT4, when it is overexpressed (Cade et al 1994). The dominant

negative effects of NOT4 alleles indicates that altering the dosage of the NOT genes can affect gene expression.

NOT3 and NOT5 were isolated as mutations that increase HIS3 transcription from the T_C element (Collart 1994; Oberholzer et al 1998). NOT3 and NOT5 show 44% identity in their amino-acid sequence, in the N-terminal region (Oberholzer 1998). Unlike the other NOT genes, deletion of NOT3 does not cause temperature sensitivity or caffeine sensitivity and has no effect on ADH2 expression (Liu et al 1998, Appendix 1). However the not3-2 allele displays the most dramatic increase in T_C dependent transcription (Collart 1994), indicating that individual components of the CCR4-NOT complex can affect the expression of different genes, dissimilarly.

Since the NOTs can distinguish between T_C and TR, the NOT proteins were suggested to restrict TBP access to non-canonical TATA sequences. Collart (1996) further showed that certain not alleles genetically interacted with MOT1 which is required for TBP removal from DNA (Auble et al 1994) and with SPT3, which binds TBP (Eisenmann et al 1992) and is required for its action at certain promoters. Although the results from most studies are consistent with the possibility that the NOTs function by regulating TBP activity, the mechanism of action of the CCR4-NOT complex remains unclear.

The focus of this dissertation is to investigate the model, that the CCR4-NOT complex functions by regulating TBP activity, and to elucidate the mechanism(s) of action of the CCR4-NOT complex

In the research presented here, I have assayed NOT function, in vivo, using strains that contain simplified HIS3 promoter derivatives containing specific mutations within the TATA sequence. Specifically I tested the effect of not mutations on activated HIS3 transcription, from mutant (non-consensus) TATA sequences that vary by a single base pair. From this analysis it was found that the NOT proteins are general repressors that reduce TBP activity irrespective of the sequence to which TBP binds. In addition, we have also obtained evidence that ccr4 , caf1, not4 deletions affect his4-912 delta expression in the same manner as certain TBP mutations. Biochemical analysis indicated that NOT2 and NOT5 can immunoprecipitate and retain TBP, $TAF_{II}130$, $TAF_{II}90$, $TAF_{II}61$, $TAF_{II}60$, $TAF_{II}40$, $TAF_{II}25$ and SPT3. Using purified complexes, it was demonstrated that NOT5 retained several components from TFIID while NOT2 associated with components of SAGA. It was also shown that TBP and TAFs displayed multiple genetic interactions with CCR4-NOT complex components. These genetic and physical interactions indicate that one role of the CCR4-NOT complex is to inhibit TBP activity, perhaps by interacting with factors which associate with TBP such as the components of TFIID and SAGA and thereby modulate their function.

MATERIALS AND METHODS

Genetic analysis

Nutrient auxotrophies and suppression of delta insertions at the HIS4 locus were scored on his-medium supplemented with the appropriate amino acids, as described in Arndt et al 1994. Phenotypic analysis of the modified HIS3 promoters was carried out by testing growth of strains on media containing 3-aminotriazole (3-AT), as described in Arndt et al 1994.

Gene disruptions and plasmids

The plasmids and gene disruption constructs used have been described previously (Liu et al 1998; Collart and Struhl 1994; Oberholzer et al 1998; Madison and Winston 1997). All the TAF_{II} overexpression plasmids used in this study were a gift from S. Buratowski and were expressed from the native promoters on 2 micron plasmids.

Immunoprecipitation

Immunoprecipitations were carried out as described previously (Draper et al 1994; Liu et al 1998) with yeast extracts prepared in Ip/wash buffer containing 50 mM phosphate, pH 7.6, 1 mM Sodium pyrophosphate, 1 mM Sodium fluoride, 1% NP40, 10% glycerol, 2 mM Magnesium chloride, 1 mM EDTA, 4 mg/L Leupeptin, 2 mg/L Pepstatin A, 1 mM Benzamidine, 1.25 mg/L Chymastatin, 1 mM PMSF. 2 mg of yeast crude extract protein in 250 microlitres of Ip/wash buffer was incubated either with 5 micrograms of anti-TBP antibody or equivivalent amount of anti-TBP pre-immune serum for 1 hour at 4°C with constant rocking. Protein A agarose beads, 25 microliters, were

added to the above mixture and incubated for 1 hour at 4°C with constant shaking. The beads were washed four times with 1 ml of the same buffer to remove unbound proteins from the yeast extract, mixed with SDS loading buffer, boiled for 5 min. and separated on a SDS-PAGE gel prior to Western analysis. The NOT5 immunoprecipitations were conducted in a similar manner

GST pulldowns

GST fusion proteins were expressed and bound to glutathione-agarose beads as described in Chiang et al 1996. Yeast whole cell extracts were prepared from the strain yEK20 containing $TAF_{II}25$-HA13 , in the same buffer used for carrying out the immunoprecipitation experiments. Washed gutathione-agarose beads were incubated with 2 mg of yeast whole cell extract protein in 250 microliters of Ip/wash buffer for 2 hours at 4oC on a rocking platform. Unbound proteins were removed by four washes with 1 ml of the same buffer and specifically bound proteins were resolved by SDS-PAGE after boiling beads directly in sample buffer. Proteins resolved by SDS-PAGE were transferred to PVDF membrane and analyzed by Western blotting as described in Draper et al 1995. Antibody against SPT3 was provided by F. Winston. $TAF_{II}25$-HA13 was detected using the 12CA5 mouse monoclonal anibody against the HA1 epitope.

GST binding

The GST fusion proteins were expressed and purified as described above. The binding protocol was similar to the protocol used in the pulldowns, except that, instead of yeast extract, the GST fusions were incubated with either 15 microliters of purified TFIID or 30 microlitrers of purified SAGA. Antibody to GCN5 was provided by S.

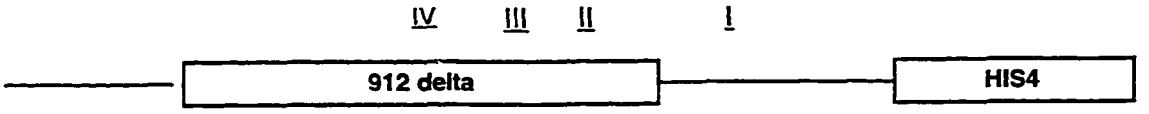
Berger. Purified SAGA was obtained as described in Sterner et al 1999 and purified $TF_{II}D$ was obtained as described in Sanders and Weil 1999. The TFIID was purified to near homogeneity and does not contain any contaminating non-TFIID TAFs such as MOT1 (Sanders and Weil 1999).The GST fusions were incubated with the purified TFIID or SAGA complex for one hour at 4°C. Following the washes as described in the GST pulldown experiments, the beads were spun down and all the supernatent was removed. 15 microliters of 5X SDS loading buffer was added to each of the samples and boiled for 5minutes.Subsequently the beads were spun down and exactly 15 microliters of each sample was loaded on to a pre-cast gradient minigel (4-20%, 0.75mm thick) from Biorad. Samples were loaded in alternate wells to prevent crossover spillage into adjacent lanes. Subsequently the proteins resolved on the SDS gradient minigel were blotted on to PVDF memebrane using a Transblotter from Idea Scientific. Subsequently the membrane was incubated in blocking solution containing 10% non-fat milk and .05% Tween 20, for one hour. Subsequently the appropriate sections of the membrane were probed with primary antibodies against specific components of TFIID or SAGA. The antibodies were incubated with the membrane overnight (12-14 hrs) at 4°C. The purified antibodies were diluted in solution containing 1% milk and .05% Tween 20 and the crude antisera were diluted in solution containing 5% milk and 1% NP40 detergent. Subsequent manipulations to visualize the proteins by ECL were carried out as described for the immunoprecipitation experiments.

RESULTS

Since the NOT proteins were found to be part of the previously identified CCR4 complex a detailed phenotypic analysis was carried out to compare the effects of ccr4, caf1 and various not deletions on expression of specific genes and on diverse cellular processes. The results from this analysis are presented in Appendix 1 (Liu et al 1998).

During this phenotypic analysis, it was observed that deletion of CCR4 causes a weak but detectable Spt- phenotype (CLD pers. comm.). SPT stands for "Suppression of Ty insertion" (Winston 1984). When transposons insert into the promoter of a gene they alter the expression of the gene. Ty elements are yeast retrotransposons that insert in and out of the genome and affect expression of various genes. Sometimes when Ty elements excise they leave behind a copy of their LTR sequence known as the "delta element". The his4-912 delta locus is a specific instance of a "delta" insertion within the HIS4 promoter that prevents functional expression of the HIS4 gene. Hence the strains are unable to grow on media lacking histidine. As seen from the Figure 2, the presence of the delta element causes the HIS4 transcript to initiate within the delta sequence. This results in the synthesis of a longer than normal transcript that is non-functional for translation due to the presence of stop codons in the 5' region of this transcript. Mutations that suppress the his4-912 delta insertion allow the HIS4 transcript to initiate from its normal start site (Arndt et al 1994).

Mutations in several genes involved in transcription have been isolated as suppressors of his4-912 delta or other Ty insertions (Winston et al 1984; Winston 1992). These include SPT15 which encodes the yeast TBP protein and genes encoding histones. Depending on the number and type of Ty insertions suppressed, the SPT genes were classified into the "TBP class" and the "Histone class of SPT genes (Winston 1992)).



III. non-consensus TATA

5' CCCTTTTATGGATTCCTA 3'
3'GGGA**AAATAC**CTAAGGAT5'

Figure 2: Schematic diagram of the his4-912 delta promoter region.

The structure of the his4-912 delta promoter is shown as described in Arndt et al 1992; 1994. The numerals I, II, III, IV, indicate the previously described (Arndt et al 1992) TBP binding sites. Site IV promotes delta expression whereas site I promotes HIS3 expression. Site III when mutated interferes with the ability of spt15-122 to suppress the his4-912 delta insertion. The sequence of site III is shown, with the residues that were mutated, indicated in bold letters.

ccr4, caf1, not4 and spt15-122 suppress his4-912 delta by similar mechanisms

Since ccr4 displayed a weak Spt- phenotype, allowing for growth on media lacking histidine, I analyzed the effect of caf1 and other not genes on his4-912 delta. I found that caf1 and not5 also exhibited a weak Spt phenotype while not4 caused a moderately strong suppression of his4-912 delta (Table 1). Since NOT1 is essential, I looked at the effect of a truncated version of NOT1 on expression from the his4-912 delta locus. It was observed that deleting the N-terminal 1300 amino acids of NOT1 did not cause an Spt-phenotype (Table 1) however it caused a dramatic decrease in the frequency of Spt-reversion (unpubl. obsv). To determine if the ccr4 and not genes belong to the TBP class of SPT genes, I analyzed expression from two other loci containing Ty insertions, which are typically suppressed only by mutations in the TBP class of SPT genes (Winston 1984; Winston 1992). I found that deleting ccr4 or not4 did not have any effect on expression from the his4-917 delta and lys2-128 delta insertion loci (data not shown), suggesting that ccr4 and not4 did not belong to the "TBP class" of SPT genes.

Since the function of the CCR4-NOT complex is thought to be related to the function of TBP(Collart 1994; Collart 1996), I further analyzed the Spt- phenotype of ccr4 and not4. I wanted to determine whether the Spt- phenotype of ccr4 and not4 is similar to that of spt15 (TBP).

spt15-122 is a mutant allele of TBP (L205F) that suppresses his4-912 delta (Arndt et al 1994) . Residue 205 is known to contact the TATA box and this mutated allele of TBP shows increased affinity for binding the non-consensus TATA sequence

TABLE 1

Ability of different mutations in the CCR4-NOT genes to suppress the his4-912 delta insertion

Genotype	Suppression of his4-912 delta	CATAA* in his4-912 delta
Wild type	-	-
ccr4	+	-
caf1	+	-
not4	++	-
not1 / dN4	-	ND
dbf2	-	ND
dhh1	-	ND
spt6	++	++
spt10	++	++
spt15-122	+	-

Growth was detected on minimal media lacking histidine. The " * " indicates that the CATAA sequence at position III within the delta element has been mutated. The spt6 data is from F. Winston, pers. comm.

"CATAAA". The ability of spt15-122 to suppress the his4-912 delta insertion depends on the "CATAAA" sequence at position III (see fig 2) within the "delta" element (Arndt et al 1994).

To further analyze the Spt- phenotype of ccr4 , caf1 and not4, we investigated the ability of ccr4, caf1 and not4 to suppress his4-912 delta in a strain wherein the CATAAA sequence at position I_{II} within the "delta" element had been mutated. We found that ccr4, caf1 and not4 were unable to suppress his4-912 delta in this strain (Table 1). In contrast spt6 and spt10, which are not part of the CCR4-NOT complex and are not functionally related to TBP (Bortvin et al 1996; Dollard et al 1994), suppressed his4-912 delta insertion even when the CATAAA sequence was mutated (Table 1 and F.Winston pers. comm.). This data suggested that the Spt- phenotype of ccr4, caf1 and not4 was mechanistically similar to the Spt- phenotype of spt15-122.

The model proposed for the mechanism by which spt15-122 suppresses his4-912 delta is that, the binding of TBP to the CATAAA sequence at position III, interferes with transcription from the " TATAA" at position IV within the delta element, thereby allowing increased transcription from the HIS4 TATAA (see Figure 2 and Arndt 1994). Our data, taken together with the above model suggests that ccr4, caf1 and not4 mutations allow increased binding of TBP to CATAAA which is a non-consensus TATA sequence. This is in agreement with previously published data that the NOT proteins function to negatively regulate HIS3 transcription from the "T_C element" which is a non-consensus TATA element (Collart 1996).

As stated earlier spt15-122 has greater affinity for CATAAA as compared to wild type TBP (Arndt et al 1994). It has also been shown that spt15-122 enables increased transcription from promoters wherein the TATAAA sequence has been replaced by CATAAA (Arndt 1994)). This analysis was done in strains wherein the T_C and TR elements in the HIS3 promoter were replaced by an oligonucleotide positioned 23 bp downstream from the GCN4 binding site (see fig. 1). The oligonucleotides were either TATAAA or derivatives of this sequence containing single base changes (Arndt et al 1994; Harbury et al 1989).Thus spt15-122 has been shown to cause increased transcription from his3-202 which is the HIS3 promoter derivative containing the CATAAA sequence instead of TATAAA.

The next question I investigated was whether ccr4 and not4 had any affect on transcription from his3-202 (CATAAA). This analysis was done using 3-aminotriazole. It has been known for several years that the level of HIS3 transcription can be assayed semi-quantitatively by the degree of resistance to 3-amino triazole (3-AT), which is a competitive inhibitor of the HIS3 gene product (Klopotowski et al 1965; Struhl 1977). Thus a strain containing his3-202 and wild type TBP is unable to grow well on 5mM 3-AT and produces low levels of HIS3 mRNA, whereas the same strain with an spt15-122 mutation produces much higher levels of HIS3 mRNA (Arndt et al 1994).

I made ccr4 , caf1, not4 and not5 deletions in the his3-202 strains and analyzed the ability to grow on media containing varying concentrations of 3-AT. As seen from Table 2, ccr4 and caf1 do not have any affect at this promoter where as not4 and not5 deletions allow growth on 20mM 3-AT, indicating that not4 and not5 deletions allow increased transcription from the CATAAA sequence. ccr4 and caf1 deletions may not show an effect at this promoter since they in general display less repressive effects than not4 or not5 (Liu et al 1998).

TABLE 2

Effect of different mutations in the CCR4-NOT

factors on his3-202 (CATAAA) expression

Genotype	5mM 3AT	10mM 3AT	20mM 3AT
wild type	w	-	-
caf1	w	-	-
ccr4	w	-	-
not4	++	+	+
not5	++	+	+

Growth was detected on minimal media lacking histidine and supplemented with varying concentrations of 3-AT as indicated in the Table. All strains are isogenic to FY664 (wild type) except for the indicated deleted allele. ++ - strong growth; + - good growth;

w – weak growth; - - no growth.

not4 and not5 deletions increase transcription from non-consensus TATA elements irrespective of sequence

Although spt15-122 and not4 and not5 have the same effect on the his3-202 promoter, it should be noted that spt15-122 is a point mutation in TBP that alters its binding specificity and increases its binding affinity for the CATAAA sequence. Therefore the effect of spt15-122 is specific to his3-202 and does not affect certain HIS3 promoter derivatives containing other substitutions in the TATAAA sequence (Arndt et al 1994).

The next question I asked was whether spt15-122 and not4 and not5 affected his3-202 (CATAAA) by similar mechanisms. This question was investigated using two approaches -

i) If not4 and not5 do not alter the binding specificity of TBP, then their effects should not be limited to one particular non-consensus TATAAA element. To test this possibility, I made not4 and not5 deletions in strains containing other HIS3 promoter derivatives. As seen in Table 3, I found that not4 and not5 deletions allowed increased HIS3 transcription from several different non-consensus TATA elements which were not affected by spt15-122.

ii) If mutations in TBP and NOT4 affected non-consensus TATA elements by the same mechanism, then combining these mutations should not have any significant additive effects.

TABLE 3

Effect of not4 and not5 deletions on different his3 promoter alleles

	TATAAG (his3-217)		TACAAA (his3-208)		TGTAAA (his3-205)	
Genotype	**5mM**	**20mM**	**5mM**	**10mM**	**5mM**	**10mM**
wild-type	-/+	-	-	-	-	-
not4	++	+	+	+	+	-
not5	++	+	+	+	+	-

Growth was monitored on minimal medium lacking histidine and supplemented with 3-AT at the concentrations indicated.

The series of HIS3 genes containing a single TATAAA-like element are all isogenic as described in Arndt et al 1994. The spt15-301 (L114F) allele has been previously shown to confer 3-AT resistance in strains containing the his3-217 (TATAAG) promoter but not to the other two his3 promoter alleles represented in this table (Arndt et al 1994)

TABLE 4

Effect of not4 and spt15-301 alleles on his3-217 (TATAAG) expression

Genotype	20mM 3AT	40mM 3AT	80mM 3AT
wild type	-	-	-
not4	+	-	-
spt15-301 (L114F)	+	+	-
not4 spt15-301 (L114F)	++	++	+

Growth was monitored on minimal media lacking histidine supplemented with 3-AT at the concentrations indicated. All the strains are isogenic to FY676 (wild type) except for the indicated alleles. ++ - strong growth; + - good growth; - - no growth.

It should be noted that, for reasons unrelated to HIS3 transcription strains containing spt15-122 mutations are very sensitive to 3-AT and hence could not be used for this analysis (Arndt et al 1994). Therefore we used spt15-301 (L114F) which is another point mutation in TBP that increases its affinity for the sequence TATAAG and thus allows increased transcription from his3-217 (TATAAG) (see Table 4 and Arndt 1994)

Thus as seen in Table 4, while not4 and spt15-301 mutations allow growth on 20mM and 40mM 3-AT respectively, in combination they allow growth on 80mM 3-AT. Thus the additive effect of these two mutations on transcription from his3-217 (TATAAG) suggests that they function by different mechanisms.

It should be noted that although certain combinations of not gene deletions display enhanced effects on growth, they do not have additive effects on HIS3 transcription (Collart 1994). However, I was unable to determine the effect of combining not4 and not5 deletions on his3-202 transcription, since combining these two deletions is synthetically lethal (Oberholzer 1998).

Taken together these data indicate that while TBP mutations alter its binding specificity and cause increased transcription from certain specific non-consensus TATA elements, the not4 and not5 mutations allow increased transcription from all non-consensus TATA elements tested. The not4 and not5 mutations appear to stabilize TBP-TATA box interactions irrespective of the sequence and by a mechanism that is apparently different from augmenting the affinity of TBP binding to a particular non-canonical TATA sequence.

As seen above, point mutations in the TATA element reduce the level of expression due to reduced stability of binding of TBP to these TATA elements. It was possible that increasing the concentration of TBP in the cell would shift the balance of the TBP-DNA binding equilibrium towards the bound form, thereby increasing the stability of this TBP-DNA interaction and allowing increased expression from the mutant TATA elements. To test this possibility, I overexpressed TBP in strains containing the his3-202 (CATAA) promoter derivative and found that it did not cause any increase in the level of HIS3 expression (data not shown). These results suggest that factors other than TBP are limiting or essential for transcription from non-consensus TATA elements. These other factors are likely to be proteins that associate with TBP and modulate its function at a subset of promoters. Such a group of proteins include the TAFs, TFIIA and SPT3, SPT8. Based on the above genetic analysis, it can be hypothesized that the CCR4-NOT complex affects TBP activity by binding and modulating the activities of one or more of these proteins. I investigated this possibility using a genetic strategy involving analysis of dosage effects and suppressors.

Dosage-dependent interactions between NOTs, TBP and TAFs

The above data implicate a balance between the CCR4-NOT complex and TBP and its associated factors in regulating gene expression. The effect of increasing the levels of CCR4-NOT protein components in combination with the TBP allele spt15-122 was subsequently investigated. Increasing the dosage of NOT1 was found to specifically inhibit the Spt- phenotype of spt15-122 (Table 5) but did not affect the

Spt- phenotype of spt10 and spt3 mutations (data not shown). Elevated expression of NOT2 or NOT4 did not have any effect in the spt15-122 strain while increasing the dosage of NOT3 caused a weak inhibition of the Spt- phenotype (Table 5). I also analyzed the effects of overexpression of TAFs in combination with not defects. I found that overexpression of $TAF_{II}19$ in a not3-2 strain and in a not5 deletion strain caused extremely slow growth at 30°C and also caused synthetic lethality at 34°C in the not5 strain (Table 6). This is a specific effect since overexpression of several other TAFs such as $TAF_{II}130$, $TAF_{II}67$, $TAF_{II}40$, did not have any effect on the growth of not3-2 and not5 strains. Also, overexpression of $TAF_{II}19$ did not have any effect in not1-2, not2, not4, or caf1 strain backgrounds (data not shown). These results indicate that the CCR4-NOT complex and TBP as well as the TAFs functionally interact with each other and that altering the balance between these two groups of proteins can result in severe growth and transcriptional defects.

TABLE 5

Effect of increased concentration of different NOT proteins on the ability of spt15-122 to suppress the his4-912 delta insertion

Increased dosage of	Effect on the ability of spt15-122 to suppress his4-912 delta
NOT1	-
NOT2	+
NOT3	w
NOT4	+
Vector alone	+

Growth was monitored on minimal media lacking histidine and uracil. The strain FY475 (spt15-122) was transformed with pRS316 derivatives containing NOT1, NOT2, NOT3, and NOT4 genes, respectively, under the control of their respective native promoters.

+ - good growth; w – weak growth; ⁻ - no growth.

TABLE 6

Effect of $TAF_{II}19$ overexpression on growth of not3-2 and not5 alleles

PLASMID	Wt		not3-2		not5	
	30°C	34°C	30°C	34°C	30°C	34°C
YEp24	+	+	+	+	+	+
TAF19	+	+	w/-	w/-	w/-	-
TAF40	+	+	+	+	+	+
TAF60	+	+	+	+	+	+
TAF67	+	+	+	+	+	+
TAF130	+	+	+	+	+	+

Growth was detected on SC plates lacking uracil, to maintain selection for the TAF_{II} plasmids. not5 (MY1735-1), not3-2 (MY25), wild type (KY803).

+ - growth; **w/-** - very weak growth; $^{-}$ - no growth. All TAF_{II} plasmids are 2 micron plasmids.

spt3, spt8 and spt15-21 mutations suppress ccr4 and caf1 deletions.

The above genetic experiments indicate that components of the CCR4-NOT complex can functionally interact with TBP and its associated factors. Although it is clear from these above experiments that the NOTs appear more closely aligned with TBP and its associated factors than CCR4 and CAF1, ccr4 and caf1 do display some genetic interaction with TBP as evidenced from the similar behavior to spt15-122 in terms of suppressing his4-912 delta. Part of this difference between CCR4 and CAF1 and the other NOT proteins may be their physical location in the CCR4-NOT complex. The not1-2 allele has been found to specifically retain the ability to interact with CCR4 and CAF1 and is most likely deficient in interacting with NOTs 2, 4, and 5 (Bai et al 1999). Since it has been shown that the temperature sensitive phenotype associated with the not1-2 allele is specifically suppressed by spt3 and spt8 deletions (Collart 1996), we therefore investigated the effect of spt3 and spt8 defects on ccr4 and caf1 phenotypes. An spt3 deletion suppressed the caffeine sensitivity and cold sensitivity of a ccr4 deletion or a caf1 deletion but did not suppress the glycerol temperature sensitivity phenotype (Table 7 and data not shown)

TABLE 7

spt3 , spt8 and spt15-21 suppress ccr4 and caf1 defects

genotype	8mM Caffeine	13°C
wt	+	+
ccr4	-	-
ccr4 spt3[a]	+	+
ccr4 spt8[b]	+	+
ccr4 spt15-21[c]	+	+
caf1	-	-
caf1 spt3[d]	+	+
spt8[e]	+	+

Growth was detected on YEP plates containing 2% glucose and supplemented with 8 mM caffeine as indicated in the Table. wt (DY3462), ccr4 (612-1d-2A), caf1 (DY3462-c1)

[a] Isogenic to 612-1d-2A except for the indicated deletion allele

[b] Analyzed four segregants of cross DY3462-4 and FY50

[c] Analyzed four segregants of cross DY3462-4 and L886

[d] Analyzed four segregants of the cross DY3462-c1 and FY464

e Analyzed four segrgants from cross DY3462-4 and FY50

An spt8 deletion also suppressed ccr4 and caf1 defects (Table 7). Using an spt15-21 allele that results in a TBP that is specifically defective in binding SPT3 (Eisenmann et al 1992), we examined whether the ability of spt3 to suppress ccr4 was related to the ability of SPT3 to bind TBP. The spt15-21 allele also suppressed defects caused by a ccr4 deletion (Table 7). An spt7 deletion did not have any affect on ccr4 and caf1 phenotypes. These results confirm that CCR4 and CAF1 also functionally interact with TBP and SPT3 and indicate that different components of the CCR4-NOT complex display different genetic interactions with TBP and its associated factors.

Overexpression of NOT1 suppressed the his4-912 delta insertion in CATAAA dependent manner

It was observed that overexpressing NOT1 from a high copy vector caused an Spt- phenotype at the his4-912 delta locus (Table 8). This effect was specific to NOT1 since overexpression of other NOTs or CCR4 did not cause an Spt- phenotype. This phenotype is not elicited by increasing the dosage of NOT1 using a low-copy centromeric vector (Table 8). Upon further analysis it was found that the ability of high copy NOT1 to suppress the his4-912 delta insertion was dependent on the presence of the CATAA sequence at position I_{II} within the his4-912 delta promoter (Table 8), as previously seen for the not4 and ccr4 Spt- effects (Table 1).

Since increased dosage of NOT1 using a low copy plasmid inhibits the ability of spt15-122 to suppress the his4-912 delta insertion (Table 5), I investigated the effect of high copy NOT1 on spt15-122 phenotypes. Consistent with the above data,

combining high copy NOT1 with spt15-122 did not inhibit the suppression of his4-912 delta (Table 9) 9). In fact it enhanced the Spt- phenotype of spt15-122 as evidenced by the better growth of spt15-122 on His- galactose media, in the presence of high copy NOT1 (Table 9).

Effects of overexpression of CCR4-NOT components on his3-202 expression

Taken together, the dependence of the Spt- phenotype of high copy NOT1 on the CATAA sequence within the his4-912 delta promoter and the additive effects of NOT1 with spt15-122 suggest that overexpressing NOT1 allows better binding of TBP to the CATAA sequence within the his4-912 delta element by a mechanism distinct from that of spt15-122. Subsequently, I analyzed the effect of overexpressing NOT1 on transcription from the his3-202 (CATAA) promoter. It was found that high copy NOT1 did not augment expression from the his3-202 promoter (Table 10). However, during this analysis I found that overexpressing NOT3 or NOT5 augmented HIS3 expression from the his3-202 (CATAA) promoter, while high copy NOT2 or CAF1 had no effect (Table 10). This result is similar to the effect of overexpressed NOT3 or NOT5 on expression from the native HIS3 promoter in a strain deleted for GCN4 (CLD pers. comm).

TABLE 8

Effect of overexpression of CCR4-NOT components on his4-912 delta expression

Plasmid	his4-912 delta suppression	CATAA* his4-912 delta suppression
NOT1	+	—
NOT2	—	ND
NOT3	—	ND
NOT5	—	ND
CCR4	—	ND
vector	—	—

Growth was detected on media lacking histidine. The strain used was DY3462. All the NOT genes are expressed from their native promoters on 2 micron plasmids containing the URA3 gene. The CCR4 plasmid was obtained by disrupting the LEU2 gene in the MPT2 plasmid (Sakai) with the URA3 gene.

TABLE 9

Effect of spt15-122 and NOT1 overexpression on his4-912 delta expression

Genotype	his4-912 delta suppression on Glucose	his4-912 delta suppression on Galactose
Wild type / vector	---	---
Wild type /Ep-NOT1	+	---
Wild type / CEN-NOT1	---	---
spt15-122 / vector	+	w
spt15-122 / Ep-NOT1	++	+

Growth was detected on minimal media lacking uracil and histidine containing glucose or galactose, as indicated. Wild type (DY3462), spt15-122 (FY475). Ep-NOT1- NOT1 is expressed from its native promoter on a 2 micron plasmid containing the URA3 gene.

CEN-NOT1- Described in Table 5 legend, NOT1 expressed from its native promoter on a centromeric plasmid.

TABLE 10

Effect of overexpression of CCR4-NOT components on expression from non-consensus TATA elements

Plasmid	his3-202 (CATAAA)		his3-217 (TATAAG)	
	5mM	10mM	5mM	10mM
NOT1	w	—	ND	ND
NOT2	w	—	ND	ND
NOT3	++	+	++	+
NOT5	++	+	++	+
CAF1	w	—	w	—
vector	w	—	w	—

Growth was monitored on minimal media lacking histidine and supplemented with 3-AT at the concentrations indicated. his3-202 (FY664), his3-217 (FY676). All the NOT genes are expressed from their native promoters on 2 micron plasmids containing the URA3 gene. The CAF1 plasmid was obtained by disrupting the LEU2 gene in the MPT0 plasmid (obtained from B. Anderson) with the URA3 gene. ++ - Strong growth;

+ - good growth; w – weak growth; — - no growth; ND – Not Done.

NOT2 and NOT5 co-immunoprecipitate with TBP

All of the above described genetic interactions and dosage effects strongly support the possibility that the CCR4-NOT complex physically interacts with TBP, TAFs or other TBP - associated factors. To initially address this possibility I carried out immuno-precipitation experiments using LexA-tagged versions of the NOT proteins. It was found that immunoprecipitating TBP with anti-TBP antibody specifically brought down LexA-NOT2 and LexA-NOT5 but did not bring down LexA alone (Figure 3A, lanes 7-8 as compared to lane 6). As an additional control, I showed that TBP pre-immune serum did not immunoprecipitate LexA-NOT2 (Figure 3A, lane 5). Immunoprecipitating TBP did not bring down LexA-CCR4, LexA-CAF1 or LexA-NOT4 (data not shown), indicating on the one hand that the LexA-NOT2 and Lex-NOT5 interaction with TBP was not due to the LexA moiety and, on the other hand, that NOT2 and NOT5 are more closely associated with TBP and its associated factors than other components of the CCR4-NOT complex.

Subsequently, it was found that immunoprecipitating TBP brought down the endogenous NOT2 (Figure 3B, lane 2), confirming that the interactions between TBP and NOT2 and NOT5 were not dependent on the presence of the LexA moiety. Anti-TBP pre-immune serum did not co-immunoprecipitate NOT2 (Figure 3B, lane 3). These data agree with recent studies indicating that NOT1 can physically associate with TBP (Lee et al 1998) and that NOT2 and NOT5 are closely associated with each other and somewhat separately from CCR4 and CAF1 in the CCR4-NOT complex (Bai et al 1999). The above observations also suggest that the immunoprecipitations

are only bringing down subsets of proteins that are associating. In order to examine TBP-NOT interactions more fully, the immunoprecipitations were conducted in the opposite direction. Immunoprecipitating NOT5 co-immunoprecipitated TBP while NOT5 pre-immune serum did not bring down TBP (Figure 4, lane 5 as compared to lane 3). Also anti-NOT5 antibody was unable to immunoprecipitate TBP from a not5 deletion strain (Figure 4, lane 4). These combined data clearly indicate that NOT5 and NOT2 can associate with TBP in vivo.

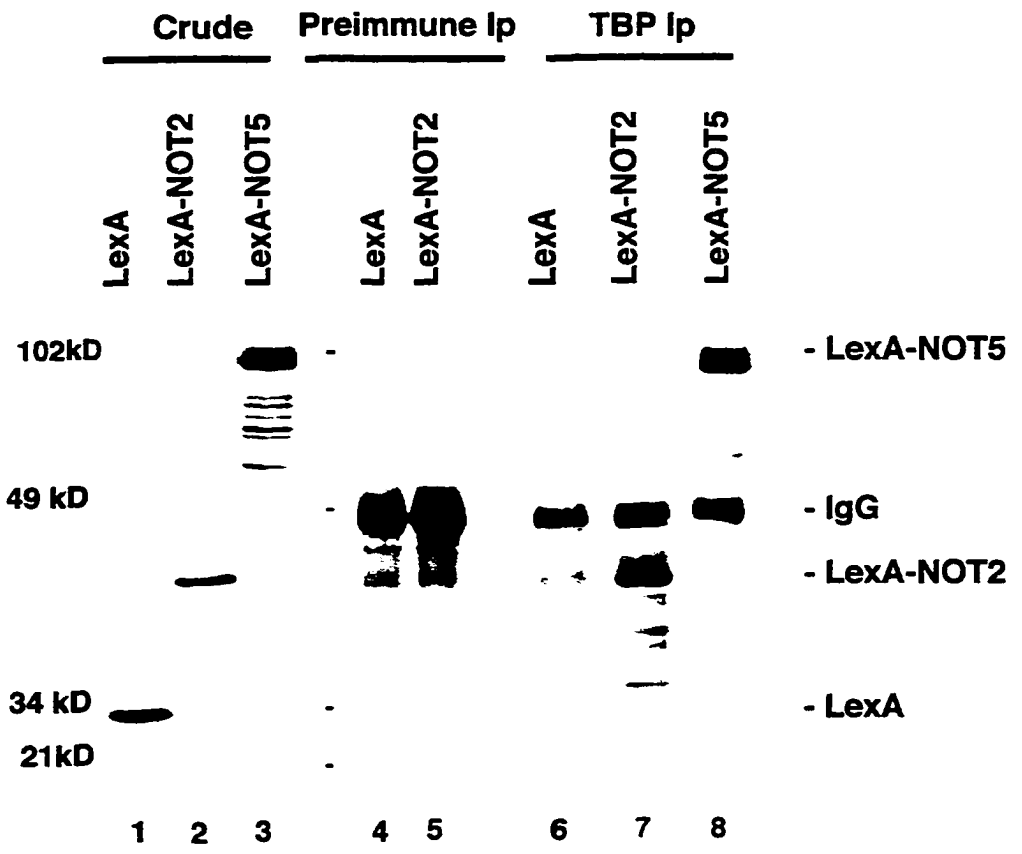


Figure 3A: TBP immunoprecipitates LexA-NOT2 and LexA-NOT5

Yeast extracts from haploid strain EGY188 containing LexA fusion proteins as indicated were immunoprecipitated with anti-TBP antibody (lanes 6-8) and with preimmune serum (lanes 4-5). LexA fusion proteins were detected by western analysis following SDS-PAGE using LexA antibody. LexA-NOT2 (lanes 2, 5, 7), LexA-NOT5 (lanes 3, 8), and LexA (lanes 1,4, 6) contained full-length NOT2, NOT5, and LexA respectively. Crude extracts are represented in lanes 1-3. 10 microliters of whole cell extract was loaded as crude extract and 250 microliters of extract was used for each immunoprecipitation reaction.

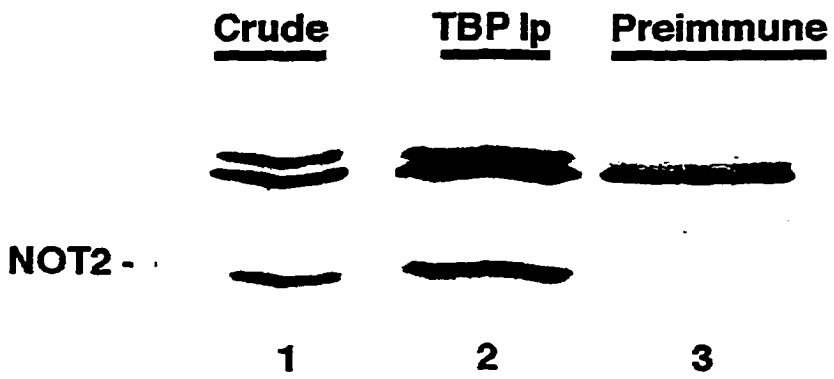


Figure 3B: TBP immunoprecipitates the endogenous NOT2 protein

Yeast extracts from strain EGY188 were immunoprecipitated with anti-TBP and preimmune serum as described in Figure 3A. NOT2 protein was detected by western analysis following SDS-PAGE using NOT2 antibody. Lane 1 represents the crude extract, lane 2 represents the anti-TBP immunoprecipitate and lane 3 represents the immunoprecipitate of the preimmune serum.

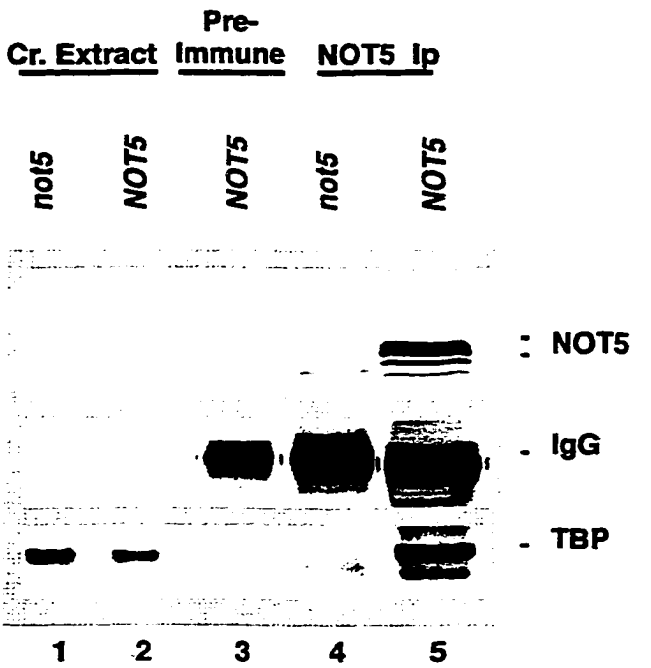


Figure 4: NOT5 immunoprecipitates TBP

Immunoprecipitations using NOT5 antiserum and preimmune serum were conducted as described in Figure 3. Lanes 1-2 represent crude extracts, lane 3 represents the preimmune serum immunoprecipitate, lanes 4-5 represent the anti-NOT5 immunoprecipitates. NOT5 - strain KY803; not5 - strain MY1735.

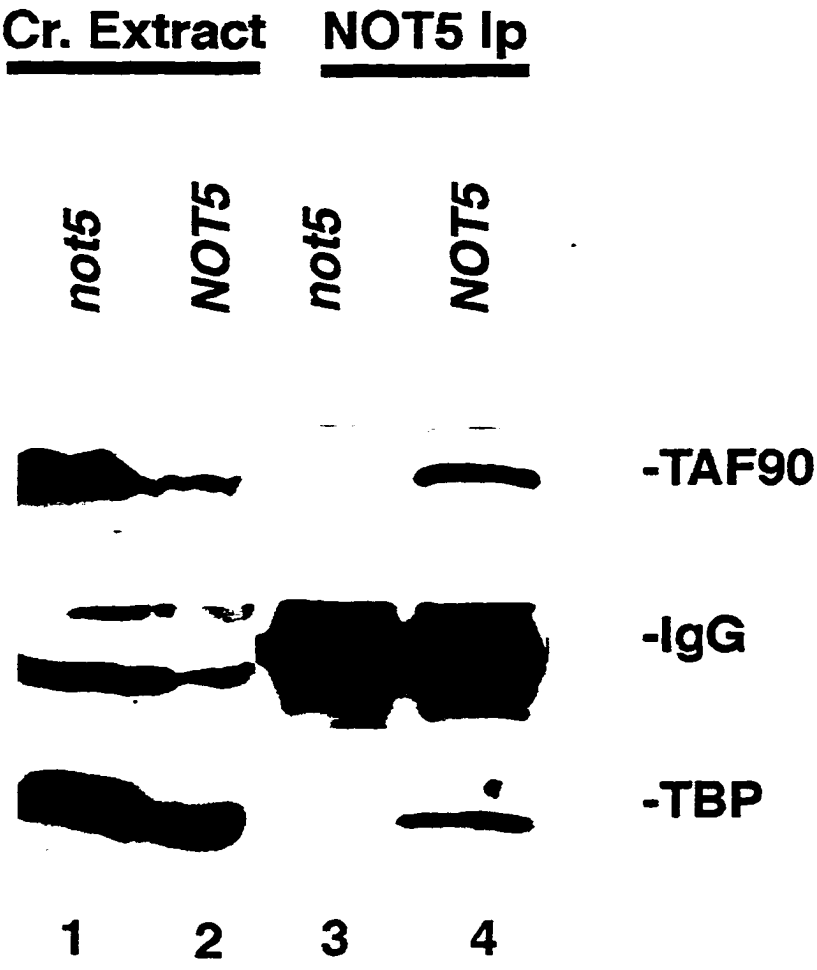


Figure 5A: NOT5 immunoprecipitates $TAF_{II}90$.

Immunoprecipitations using NOT5 antiserum and subsequent analysis were performed as described in Figure 4. NOT5 immunoprecipitates $TAF_{II}90$. Lanes 1 and 2, Crude extract.Lanes 3 and 4, NOT5 immunoprecipitations.Lanes 2, 4 - Strain KY803. Lanes 1, 3 - Strain MY1735. Western analysis was done with anti-$TAF_{II}90$ and TBP antiserum. IgG is also indicated.

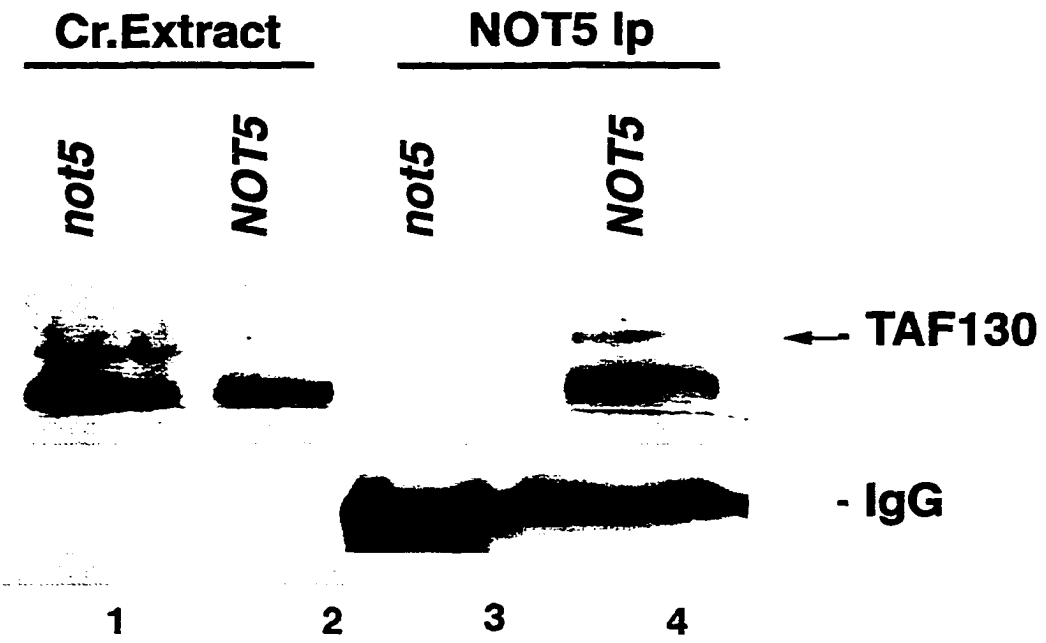


Figure 5B: NOT5 immunoprecipitates $TAF_{II}130$.

Immunoprecipitations using NOT5 antiserum and subsequent analysis were performed as described in Figure 4. NOT5 immunoprecipitates $TAF_{II}130$. The arrow indicates the position of the full length $TAF_{II}130$ protein and the band below that is presumed to be $TAF_{II}130$ degradation product. Lane designations are same as in "5A" above. NOT5 - strain KY803; not5 - strain MY1735.

NOT2 and NOT5 associate with several TAFs

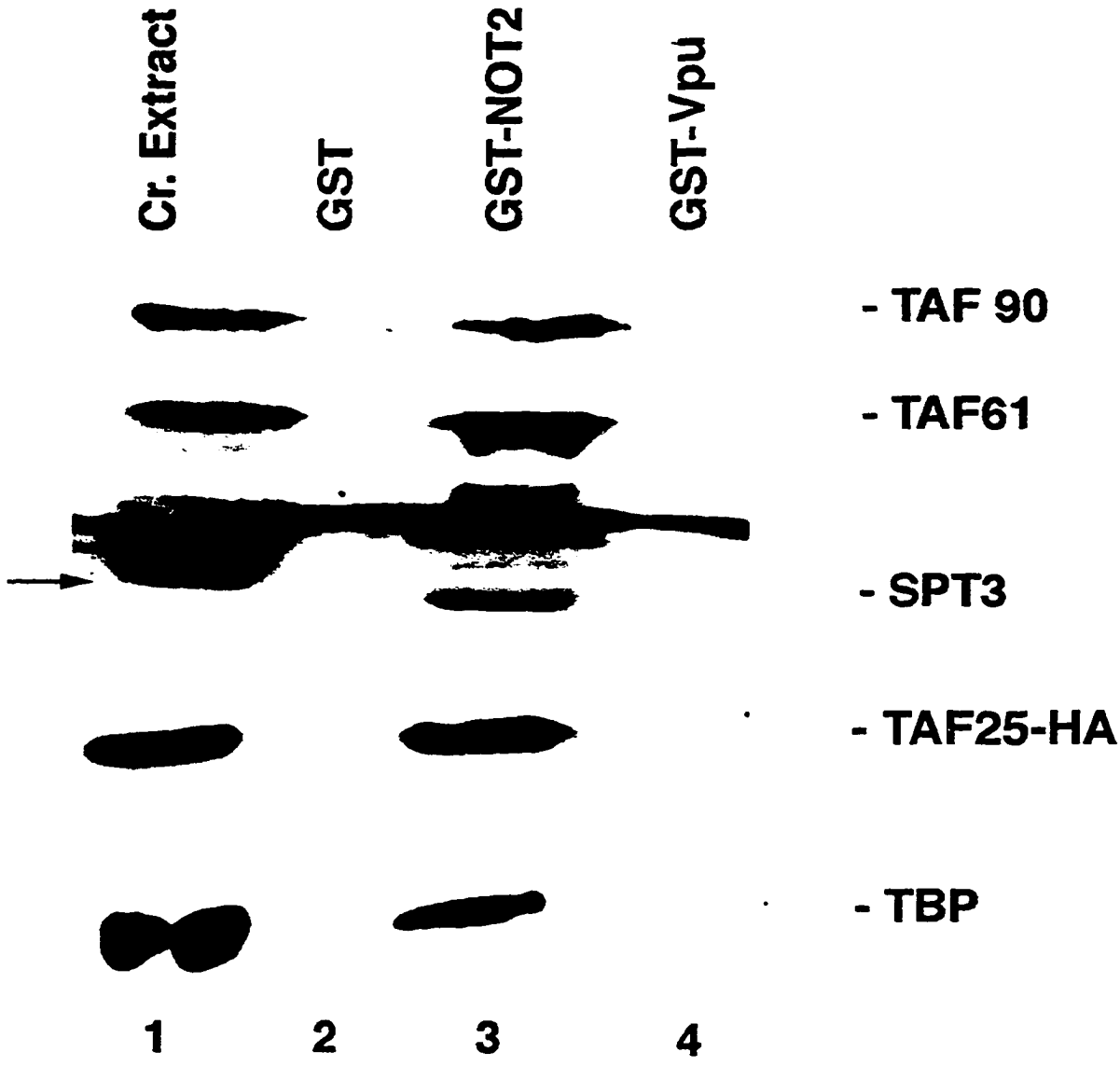
Since TBP co-immunoprecipitated with NOT5, I subsequently probed for the presence of TAFs in the NOT5 immunoprecipitate. It was found that both $TAF_{II}90$ and $TAF_{II}130$ specifically co-immunoprecipitated with anti-NOT5 antibody from a wild type strain but not from a strain deleted for NOT5 (Figure 5A and 5B, lanes 3 and 4 respectively). It should be mentioned that, in contrast to the above results, immunoprecipitating CCR4 did not bring down TBP or $TAF_{II}90$ (Komarnitsky et al 1998; data not shown). This result is expected since anti-CCR4 antibody appears to only immunoprecipitate the CCR4-NOT complex (Draper et al 1994; Liu et al 1998; unpubl. observe.) and LexA-CCR4 was not immunoprecipitated with anti-TBP antibody. It should be noted that some of the other TAF_{II}s (such as $TAF_{II}61$ and $TAF_{II}60$) non-specifically immunoprecipitate with the anti-NOT5 antibody (data not shown) and therefore their specific immunoprecipitation with NOT5 could not be determined.

To analyze these interactions from another direction I analyzed the association of TBP and its associated factors with the CCR4-NOT complex protein components using GST-NOT2 and GST-NOT5 proteins that were expressed and purified from E. coli (see Figure 6B for the relative levels of GST-NOT2 and GST-NOT5 and the controls GST and GST-Vpu used in these experiments). When whole cell extracts from wild type yeast strains were passed over these GST fusions, GST-NOT2 specifically retained $TAF_{II}90$, $TAF_{II}61$, $TAF_{II}25$-HA and TBP while the control GST fusions did not retain any of the above mentioned proteins (Figure 6A, lane 3 as

compared to lanes 2 and 4). Since the TAFs specifically retained by GST-NOT2 are also components of the SAGA complex I probed for SPT3 which is present only in the SAGA complex but is not a component of TFIID. It was found that SPT3 was also specifically retained by GST-NOT2 (Figure 6A, lane 3). In similar experiments GST-NOT5 retained $TAF_{II}90$, $TAF_{II}61$, $TAF_{II}40$, TBP (Figure 7A, lane 3) and SPT3 (Figure 7B lane3).

Figure 6A: GST-NOT2 retains TBP, SPT3, and several TAF_{II}s.

GST fusions were induced as described (Chiang et al 1996), bound to glutathione agarose beads, and incubated with yeast crude extract from the strain yEK20 containing TAF_{II}25-HA13 as described in "Materials and Methods". Proteins were eluted from glutathione-agarose beads by boiling and separated by SDS-PAGE. Western analysis was conducted by probing with appropriate antibodies. TAF_{II}25-HA13 was detected using the 12CA5 mouse monoclonal antibody against the HA1 epitope.Lane 1- crude extract; lane 2 - GST; lane 3 - GST-NOT2; lane 4 - GST-Vpu. The lane 1 SPT3 band is indicated with an arrow.



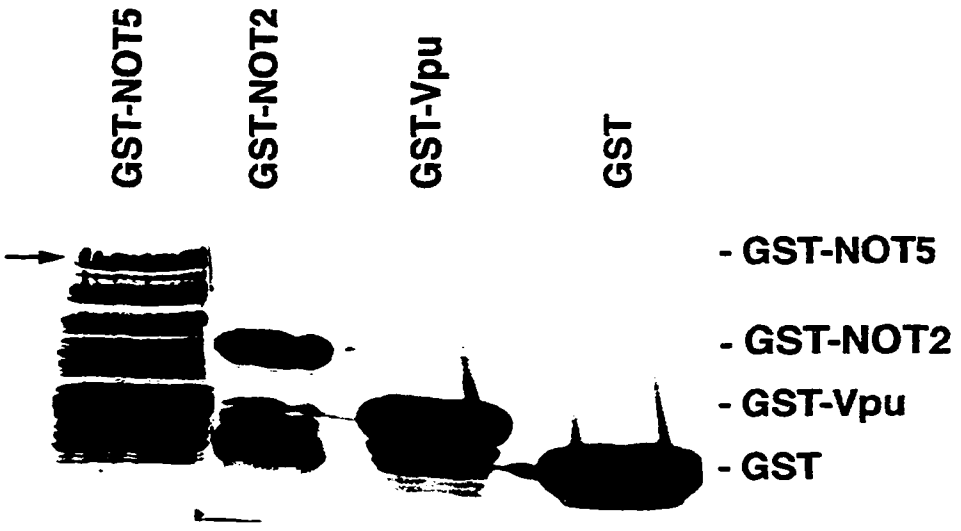


Figure 6B: Coomassie Blue stained gel showing expression levels of GST-fusions

Protein levels of the GST fusions used in Figure 6A and 7, as determined by Coomassie Blue staining. The bands beneath GST-NOT5 (arrow) are apparent degradation products as determined by Western analysis.

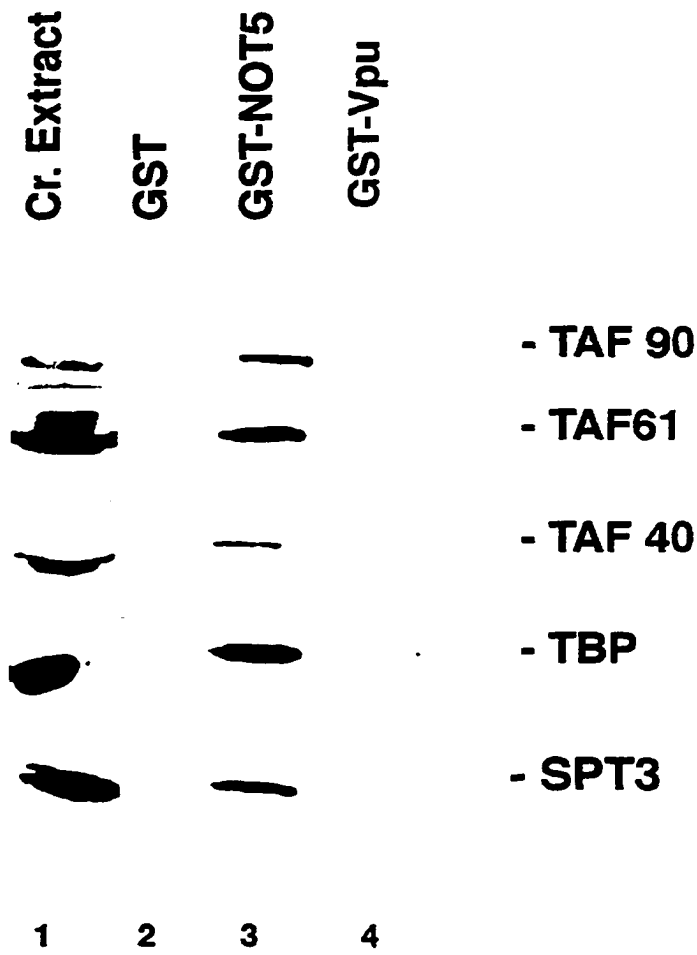


Figure 7: GST-NOT5 retains TBP, SPT3, and several TAF_{II}s.

GST pulldown experiments were performed as described in Figure 6A. Lane 1 - crude extract; lane 2 - GST; lane 3 - GST-NOT5; lane 4 - GST-Vpu.

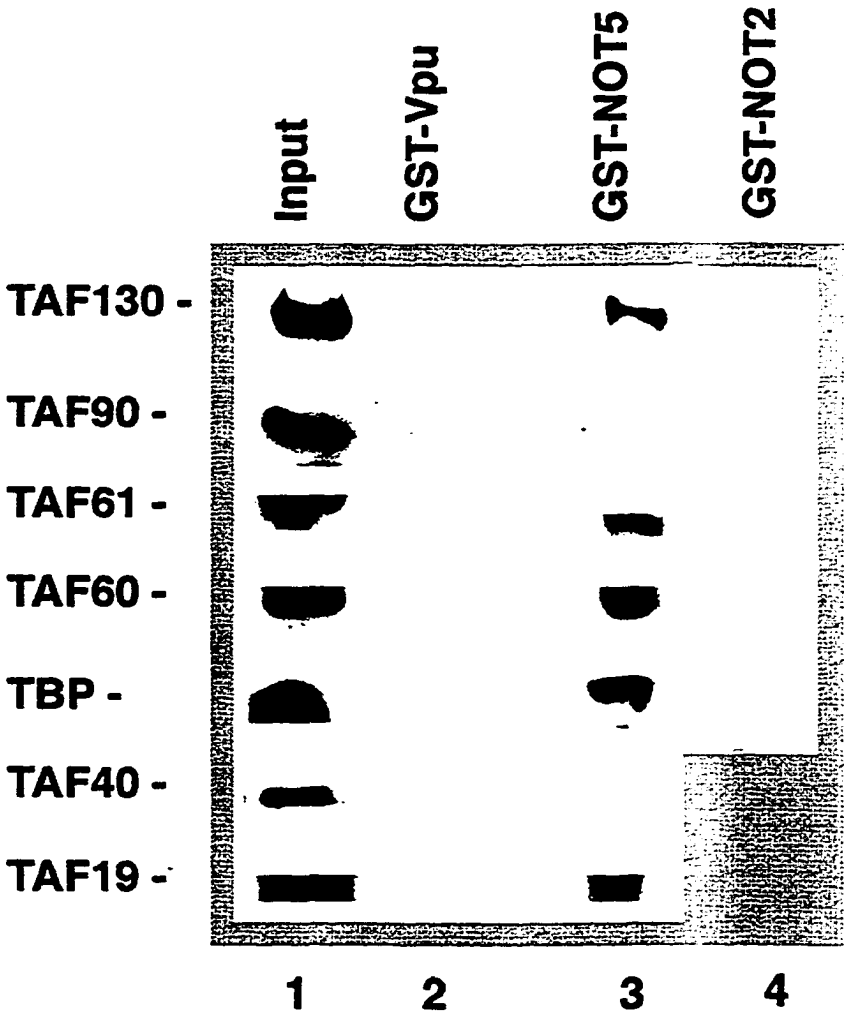


Figure 8: Binding of purified TFIID with GST-NOT5 and GST-NOT2

The GST fusions were incubated with 15 microliters of purified TFIID for one hour at 4 oC. Further manipulations were carried out as described in Figure 6A. Lane 1- Input, 5 microliters of TFIID; Lane 2- GST-Vpu; Lane 3- GST-NOT5; Lane 4- GST-NOT2. $TAF_{II}40$ and $TAF_{II}19$ antibodies were not used to probe the interactions of GST-NOT2 with purified TFIID.

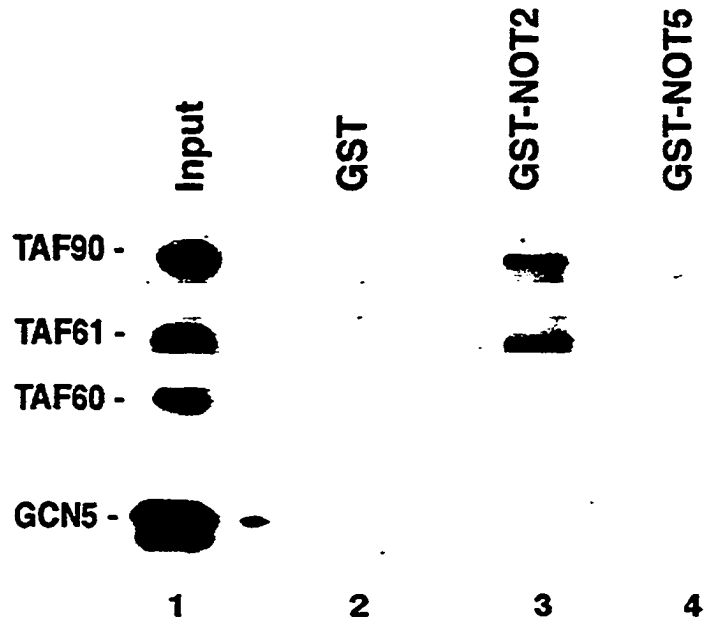


Figure 9: Binding of purified SAGA with GST-NOT2 and GST-NOT5

The binding was carried out as described in Sterner et al 1999. The GST fusions were incubated with 30 microliters of purified SAGA. Further manipulations were carried out as described in Figure 6A. Lane1- Input, 10 microliters of SAGA; Lane 2- GST; Lane 3- GST-NOT2; Lane 4- GST-NOT5.

NOT5 and NOT2 retain multiple components from purified TFIID and SAGA, respectively

The above results indicated that NOT2 and NOT5 could associate with several proteins that were components of TFIID and SAGA or both. To further dissect these interactions I carried out binding experiments using purified TFIID and purified SAGA complexes. It was found that GST-NOT2 did not retain any of the proteins tested from purified TFIID (Figure 8 lane4). In contrast GST-NOT5 retained $TAF_{II}130$, $TAF_{II}61$, $TAF_{II}60$, TBP and $TAF_{II}19$ from purified TFIID but $TAF_{II}90$ and $TAF_{II}40$,were not retained by GST-NOT5 (Figure 8 lane3). In similar experiments with purified SAGA I found that GST-NOT5 did not retain any of the proteins tested (Figure 9 lane4) while GST-NOT2 retained $TAF_{II}90$ and $TAF_{II}61$ but $TAF_{II}60$ was not retained by GST-NOT2 (Figure 9 lane 3).

Genetic and biochemical interaction between TFIIA and the CCR4-NOT complex

It has been shown that overexpression of TBP specifically enhances transcription from consensus TATA elements but not from non-consensus TATA elements (Colgan et al 1992; Collart 1996). This indicates that one or more factor(s) other than TBP are important for transcription from non-consensus TATA elements. It is possible that the NOT proteins function by blocking access of these factor(s) to the promoter or by inhibiting the function of these factor(s). Based on this hypothesis it should be possible to overcome the repressive effects of the NOTs by increasing the concentration of these factor(s).

Since TAFs, TFIIA and SPT3 are factors that associate with and modulate TBP function positively, one or more of these factors could potentially be required for optimal function of TBP at non-consensus TATA elements. I analyzed if overexpression of any of these proteins augmented expression from the his3-202 (CATAA) promoter (data not shown). Among all the proteins analyzed, only overexpression of TFIIA caused increased expression from the his3-202 (CATAA). TFIIA consists of two subunits TOA1 and TOA2. Overexpression of each subunit individually did not affect expression from the his3-202 promoter (Table 11). This suggests the the effect is dependent on the function of TFIIA and is not due to titration of any factor that binds one of the subunits of TFIIA. Subsequently I found that overexpression of TFIIA augmented expression from his3-217 (TATAAG) (Table 11) and his3-216 (TATAAT), indicating that increasing the levels of TFIIA increases expression from several different non-consensus TATA elements irrespective of their sequence. This was similar to what was observed when not4 and not5 deletions were tested (Table 2 and 3).

TABLE 11

Effect of overexpressing TFIIA subunits on different his3 promoter alleles

Plasmid	his3-202 (CATAAA) 20 mM	his3-217 (TATAAG) 20 mM
vector	—	—
TFIIA	+	+
TOA1+ TOA2	+	+
TOA1(Y69A) + TOA2	---	---
TOA1	—	ND
TOA2	—	ND

Growth was monitored on media lacking histidine supplemented with 3-AT at the concentrations indicated. his3-202 (FY664), his3-217 (FY676). The TFIIA plasmid expresses both subunits of TFIIA and has been described in Madison andWinston 1998.

TOA1 and TOA2 genes are expressed from their native promoters on 2 micron plasmids containing the LEU2 and URA3 genes, respectively.

Subsequently, I investigated whether deletion of not4 and overexpression of TFIIA augmented expression from non-consensus promoters by affecting the same step in transcription initiation at these promoters. I overexpressed TFIIA in strains deleted for not4 and containing the his3-202 allele and found that there was no additive increase in expression from the his3-202 promoter (Table 12). Similar results were obtained when TFIIA was combined with a not5 deletion (Table 12). This data suggested that overexpression of TFIIA and deletion of not4 or not5 augmented expression from non-consensus TATA elements by affecting the same step in transcription initiation at these promoters. Presumably this step involves the functional interaction between TBP and TFIIA. Subsequently I analyzed if TBP-TFIIA interactions were important for the effects of TFIIA on expression from the non-consensus TATA elements. This analysis was performed using a mutant allele of the TOA2 containing the Y69A substitution. This particular allele is defective for interaction between TFIIA and TBP and has been shown to reduce expression of several genes, including HIS3 (Ozer et al 1998). In contrast to the wild type subunits, the Y69A allele of TOA2, when overexpressed in combination with TOA1, did not augment expression from the his3-202 (CATAA) promoter (Table 11).Therefore the ability of high copy TFIIA to increase expression from non-consensus TATA elements requires functional interaction between TFIIA and TBP.

TABLE 12

Effect of TFIIA and not deletion on expression from his3-202 (CATAAA)

Genotype	10 mM	20 mM	40 mM
Wild type/ TFIIA	+	+	—
not4	++	+	w
not4 / TFIIA	++	+	w
not5	++	+	w
not5 / TFIIA	++	+	w

Growth was monitored on minimal media lacking histidine supplemented with 3-AT at the concentrations indicated. Wild type (his3-202, FY664). All strains are isogenic derivatives of FY664. The TFIIA plasmid expresses both subunits of TFIIA and has been described in Madison andWinston 1998.

TABLE 13A

Effect of combining not4 deletion with overexpression of TOA alleles on his3-202 (CATAAA) expression

Genotype	5 mM	10 mM	20 mM	40 mM
not4 / vector	++	++	+	w
not4 / TOA1	++	++	+	w
not4 / Y69A	+	w	—	—

Growth was monitored on media lacking histidine supplemented with 3-AT at the concentrations indicated. not4 (FY664-n4). TOA1 alleles are expressed from their native promoters on 2 micron plasmids containing the LEU2 gene. Vector (YEp13)

TABLE 13B

Effect of TOA1(Y69A) overexpression on expression from his3-202 (CATAAA)

Genotype	2 mM	5 mM
Wt / vector	+	w
Wt / TOA1	+	w
Wt / Y69A	w	—

Growth was monitored on media lacking histidine supplemented with 3-AT at the concentrations indicated. Wt (his3-202, FY664).

Thereafter I investigated the role of TBP-TFIIA interactions in the effects of not deletions on expression from the non-consensus TATA elements. I found that overexpressing the Y69A allele of TOA2 reduced expression significantly from the his3-202 promoter in a not4 deletion strain (Table 13A). This indicated that the Y69A allele of TOA2 had a dominant negative effect by replacing the wild type TOA2 subunit in the TFIIA dimer, thereby disrupting TBP-TFIIA interactions. The reduction in his3-202 expression suggests that functional TFIIA-TBP interactions are required for the not4 deletion mediated effects on expression from non-consensus TATA elements. However high level expression of the Y69A allele of TOA2 was found to cause a further decrease in the low level expression from the his3-202 (CATAA) promoter in the wild type strain (Table 13B). This result raises the possibility that the TFIIA functions downstream of the NOTs and that TFIIA is not directly involved in the mechanism of action of the CCR4-NOT complex.

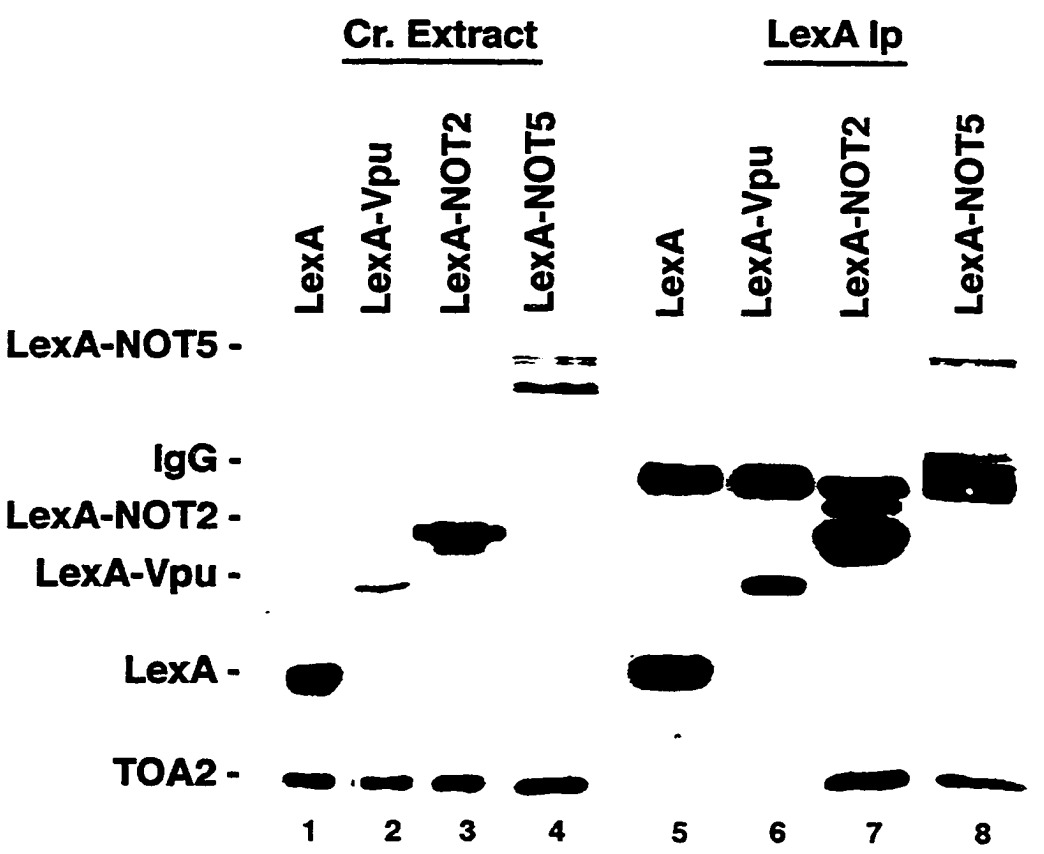
A biochemical approach was taken to further explore the possible role of TFIIA in the functioning of the CCR4-NOT complex. This work was carried out in collaboration with Y-C. Chiang a research scientist in our lab. Using the type of immunoprecipitation experiments previously described, it was found that immunoprecipitating LexA-NOT2 or LexA-NOT5 specifically coimmunoprecipitated TOA2, while neither the control LexA-Vpu nor the LexA moiety by itself co-immunoprecipitated TOA2 (Figure 10). TOA1 could not be detected due to the high background signal from the rabbit IgG which migrates at the same position as TOA1. TOA2 was also found to specifically co-immunoprecipitate with the endogenous

NOT5 protein, when immunoprecipitated using anti-NOT5 antibody (Figure 11). In contrast, immunoprecipitating CCR4 did not co-immunoprecipitates TOA2 .

These results indicated a physical interaction between NOT5 and TFIIA. The interaction between TBP and TFIIA is well documented and I had observed a physical association between TBP and NOT5 as well as NOT2. To further dissect these interactions we utilized the previously described Y69A allele of TOA2. TBP immunoprecipitations were carried out from a strain containing only the Y69A allele of TOA2 and from the isogenic wild type strain. It was found that, while TBP could co-immunoprecipitate TOA2 from the wild type strain, no interaction between TBP and TOA2 was detected in the Y69A strain (Figure 12, lane 3 vs 4). In similar immunoprecipitation experiments using antibody against NOT5, it was observed that NOT5 could co-immunoprecipitate TBP and TOA2 from the wild type as well as from the Y69A strain (Figure 13, lanes 3 and 4). Taken together these results indicate that the interaction between NOT5 and TBP is not mediated by TFIIA. Moreover, these results imply that the TBP-NOT5 and the TFIIA-NOT5 interactions occur independent of each other.

Figure 10: LexA-NOT2 and LexA-NOT5 immunoprecipitate TFIIA

Yeast extracts from haploid strain EGY188 containing LexA fusion proteins as indicated were immunoprecipitated with anti-LexA antibody (lanes 5-8). LexA fusion proteins were detected by western analysis following SDS-PAGE using LexA antibody. LexA-NOT2 (lanes 3, 7), LexA-NOT5 (lanes 4, 8), LexA (lanes 1, 5) and LexA-Vpu (lanes 2, 6) contained full-length NOT2, NOT5, LexA and LexA-Vpu respectively. Crude extracts are represented in lanes 1-4. 10 microliters of whole cell extract was loaded as crude extract and 250 microliters of extract was used for each immunoprecipitation reaction.



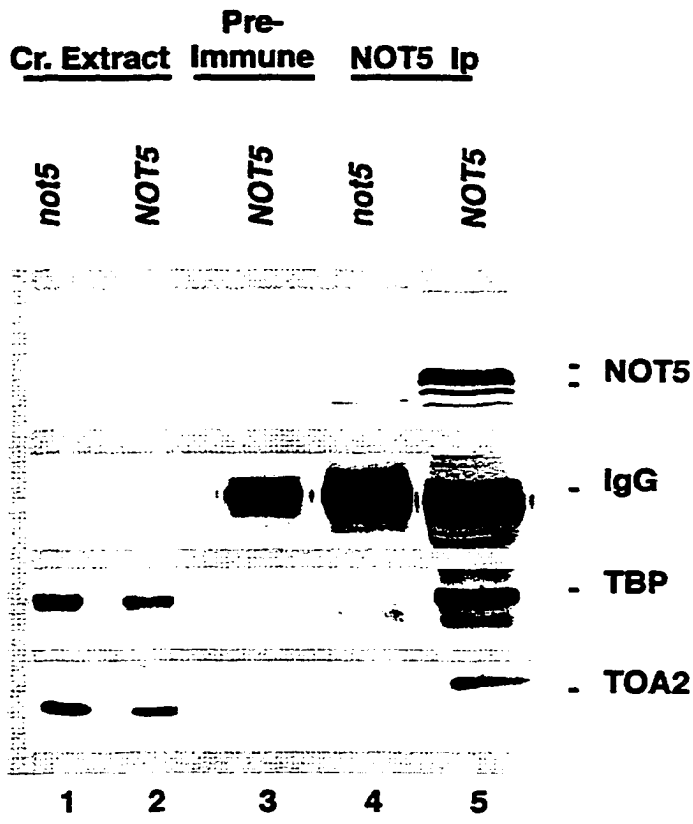


Figure 11: NOT5 immunoprecipitates TFIIA

Immunoprecipitations using NOT5 antiserum and preimmune serum were conducted as described in Figure 4. Lanes 1-2 represent crude extracts, lane 3 represents the preimmune serum immunoprecipitate, lanes 4-5 represent the anti-NOT5 immunoprecipitates. NOT5 - strain KY803; not5 - strain MY1735.

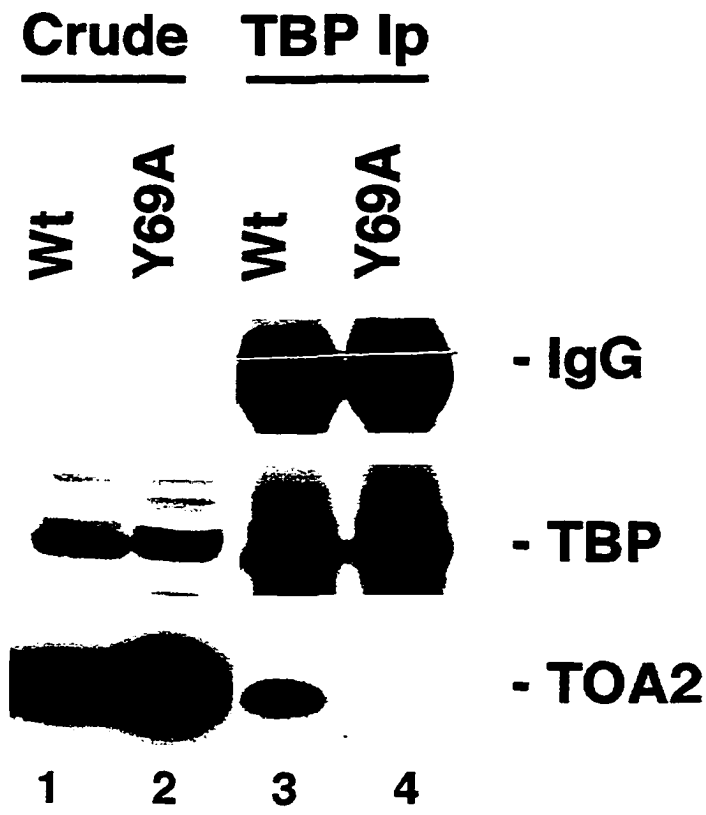


Figure 12: TBP does not immunoprecipitate TFIIA from the TOA2 (Y69A) strain

TBP immunoprecipitations were carried out as described in Figure 3. The immunoprecipitations were carried out using extracts prepared from strains Y61 (Wt) and Y69 (toa2-Y69A). Lanes 1-2 represent crude extracts, lanes 3-4 represent the anti-TBP immunoprecipitates.

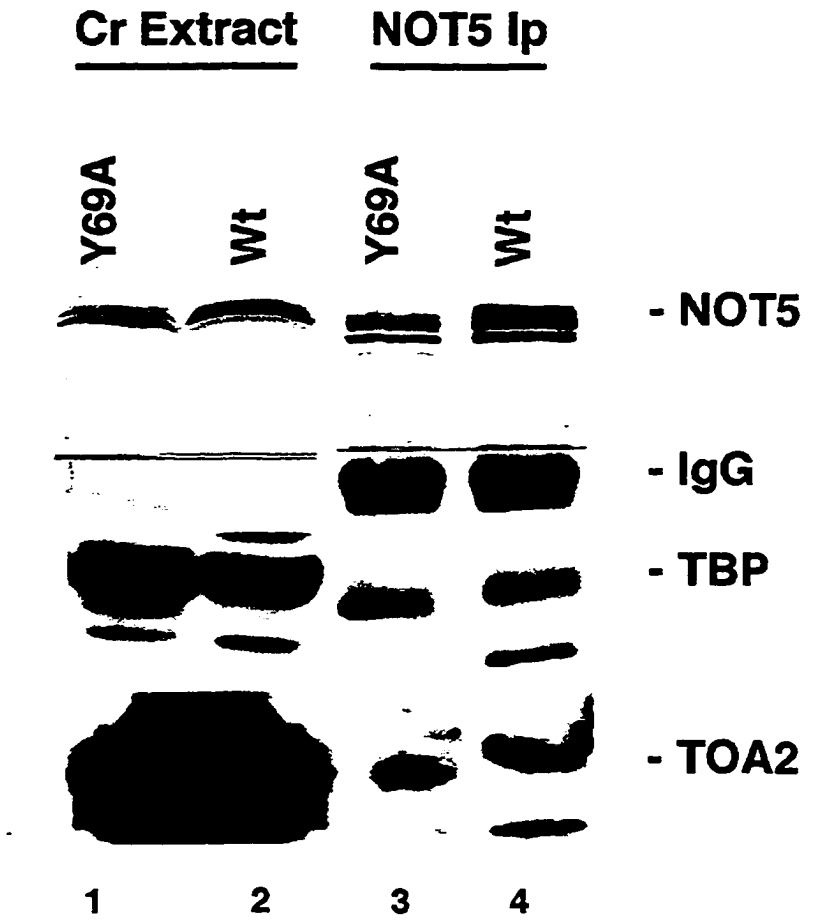


Figure 13: NOT5 immunoprecipitates TBP and TFIIA from the TOA2 (Y69A) strain

Immunoprecipitations using NOT5 antiserum were conducted as described in Figure 4. The immunoprecipitations were carried out using extracts prepared from strains Y61 (Wt) and Y69 (toa2-Y69A). Lanes 1-2 represent crude extracts, lanes 3-4 represent the anti-NOT5 immunoprecipitates.

DISCUSSION

Functional interaction between the CCR4-NOT complex with TBP and associated factors

These studies have demonstrated the functional and physical interactions between the CCR4-NOT complex and that of TBP and its associated factors . The functional interaction between TBP and the CCR4-NOT complex is based on four lines of evidence. Firstly, mutations in CCR4, NOT4, and NOT5 suppress the his4-912 delta insertion by a mechanism similar to that observed for the mutated TBP allele spt15-122. This mechanism appears to involve stabilization of TBP binding to a specific non-consensus TATA sequence, CATAAA, in the his4-912 delta element. Secondly, a specific mutation in TBP (L205F) and deletion of not2, not4, and not5 all caused increased transcription from modified HIS3 promoters containing specific non-consensus TATA elements. Thirdly, increased dosage of NOT1 specifically inhibited the ability of spt15-122 to suppress the his4-912 delta insertion but did not affect the Spt- phenotype of spt3 and spt10 at this locus. Fourthly, spt3, spt8, and spt15-21 alleles (all involved in affecting interaction of SPT3 with TBP) suppressed ccr4 and caf1 defects. These results establish a functional relatedness between the CCR4-NOT complex and TBP and its associated factors that had only been previously suggested (Collart 1996).

It was also shown that the function of CCR4 and CAF1 is closely linked to the function of NOT1, since mutations in all three genes can be suppressed by spt3, spt8

and by a specific mutation in TBP, spt15-21, that blocks TBP-SPT3 interactions (Eisenmann 1994). This result indicated that ccr4, caf1 and not1-2 mutations had the same effect at certain promoters and that functional interaction between TBP and SPT3 is essential for optimal transcription from these promoters. These results also confirm the previous close physical and functional association between CCR4, CAF1 and the NOT1 proteins (Draper et al 1995;Liu et al 1998; Bai et al 1999).

ccr4, caf1, not4, and spt15-122 suppress his4-912 delta expression by similar mechanisms

It was demonstrated that mutations in certain components of the CCR4-NOT complex cause suppression of the his4-912 delta insertion by a mechanism similar to that proposed for spt15-122. The model proposed for the mechanism by which spt15-122 suppresses his4-912 delta is that the binding of TBP to the CATAAA sequence at position I_{II} interferes with transcription from the " TATAA" at position IV within the delta element, thereby allowing increased transcription from the HIS4 TATAA (see Figure 2) (Arndt et al 1994). Our data, taken together with the above model suggests that ccr4, caf1, and not4 mutations also allow increased binding of TBP to CATAAA, a non-consensus TATA sequence. This model is consistent with the previously proposed role of NOT proteins in repressing the non-consensus TATA element, T_C, dependent transcription from the HIS3 promoter (Collart 1996).

Mutations in NOT genes cause increased transcription from non-consensus TATA elements irrespective of the sequence

Subsequently it was found that mutations in the not genes allow increased transcription from several different "mutant" TATA sequences which could be classified as non-consensus TATA elements. The unique and distinguishing feature of this analysis is that it was carried out in strains in which the HIS3 promoter was modified such that it contained only one TATA element, expression from which was activated by a wild-type allele of GCN4. The results from these studies indicate that the function of the NOT proteins is not specific to the T_C element . Rather, the NOT proteins are general repressors of TBP function, irrespective of the DNA sequence bound by TBP. This model is also consistent with the role of the NOT proteins as global repressors of transcription, as inferred from the fact that mutations in CAF1 and NOT genes can suppress mutations in SRB4 and RPB1, both of which are essential for global mRNA synthesis (Lee et al 1998).

However, the mechanism by which not alleles allow increased transcription from these non-consensus TATA sequences appears different than altering the binding per se of TBP to the 'TATA'- like sequences. This is suggested by two observations. First, the expression from several different ' TATA'- like sequences was augmented by not alleles. In contrast, TBP alterations such as L205F or L114F tend to only affect expression from one or another ' TATA'-like sequence (Arndt et al 1994). Therefore, it is unlikely that the not deletions result in altered binding

specificity of TBP. Second, a not4 deletion caused increased 3-AT resistance even in a TBP (L114F) background, suggesting that the not effect occurred by a mechanism separate from that of L114F effects on the DNA binding specificity of TBP.

Mechanism of action of CCR4-NOT complex in affecting TBP function

The genetic analysis described above and previous data (Collart and Struhl 1993, 1994; Oberholzer et al 1998), support the model that the NOT proteins exert their repressive effects by destabilizing functional interaction of TBP with DNA. The NOT proteins could accomplish this by several possible mechanisms. 1) The NOT proteins could directly bind TBP and inhibit its ability to bind DNA. This possibility seems unlikely in that, as described above, the not alleles affected functional TBP binding to a variety of non-consensus TATA sequences and by a mechanism that is apparently separate from TBP binding to DNA. This model is also inconsistent with the fact that overexpression of TBP did not overcome the repressive effects of the NOTs at the modified HIS3 promoters used in these studies (unpub. observe.). This implies that TBP direct binding to DNA by itself is not being affected. 2) It is possible that the NOT proteins bind directly to TBP and prevent the interaction of TBP with other stabilizing factors like TFIIA and SPT3. This possibility is also unlikely since no direct interaction was detected between TBP and any of the NOT proteins tested (K. Reed and C L. Denis, unpub. observe.). 3) The NOT proteins might sequester certain factors such as TFIIA, SPT3, or TAFs, that function to stabilize TBP -DNA interactions. 4) The NOT proteins may also destabilize the TBP - DNA interaction by multiple mechanisms. This latter possibility is consistent with the

fact that mutations in different not genes confer overlapping but not identical phenotypes (Liu et al 1998; Collart et al 1996; Oberholzer et al 1998). Furthermore, the fact that the spt3 deletion can suppress ccr4, caf1 and not1-2 defects but cannot suppress not1-1 and not4-1 defects (Collart 1996) suggests that these two different classes of mutations affect different promoters or affect the same promoters but by different mechanisms.

Dosage dependent interactions between NOTs, TBP, and TAF_{II}s

Overexpression of $TAF_{II}19$ in a not5 or not3-2 background gave a synthetic lethal or sick phenotype. Overproducing other TAFs did not elicit this phenotype nor did $TAF_{II}19$ display this phenotype in ccr4 or caf1 deleted strains. $TAF_{II}19$ depletion from the cell has also been shown to result in specific decreased 'TATA-less' transcription from the HIS3 locus (Moqtaderi et al 1996), suggesting that it may be involved in processes affected by the NOT proteins. In addition, increasing the dosage of NOT1 (on a low copy vector), but not any of the other CCR4-NOT components, specifically inhibited the Spt- phenotype of the spt15-122 allele. On the other hand, increasing the expression of NOT1 using a high copy vector caused an Spt- phenotype. Overexpression of any other component of the CCR4-NOT complex did not suppress the his4-912 delta insertion. Similar to what was observed for spt15-122, ccr4, not4, mutations, the Spt- phenotype caused by overexpression of NOT1 was dependent on the presence of the CATAA sequence within the delta element (Figure 2). One possibility that is consistent with the previously described mechanism is that , the overexpressed NOT1 binds some component(s) of the CCR4-NOT

complex (such as not4 or caf1) and sequesters it resulting in a condition that mimics the deletion of that component(s). These genetic interactions suggest that there exists a dosage dependent interaction between the CCR4-NOT proteins, TBP, and pol II TAFs, an interaction that is consistent with several of the above described models for CCR4-NOT function.

Components of the CCR4-NOT complex physically interact with TBP and associated factors

This model predicts that the CCR4-NOT complex or components therein should physically interact with TBP and/or its associated factors. I have found that NOT2 and NOT5 do indeed co-immunoprecipitate with TBP. This co-immunoprecipitation occurred regardless of whether TBP was first immunoprecipitated or NOT5 was first immunoprecipitated. Relatedly, NOT1 was shown to be retained from crude extracts by GST-TBP (Lee et al 1998). I failed, however to detect NOT4, CCR4, and CAF1 in our TBP immunoprecipitates. These differences may derive from the physical associations observed within the CCR4-NOT complex (Figure 14 and Bai et al 1999). We found that NOT2 and NOT5 can associate independently of CCR4 and CAF1 and that NOT2 and NOT5 associate with a different part of NOT1 than do CCR4 and CAF1 (Bai et al 1999). These observations suggest that NOT2 and NOT5 could function independent of CCR4 , CAF1, and perhaps NOT4. The synthetic lethality of not4 and not5 double deletions (Oberholzer et al 1998), further suggests that NOT4 and NOT5 proteins have different roles and therefore separate functional and physical interactions in the

CCR4-NOT complex. The immunoprecipitation data is also consistent with the fact that ccr4 and caf1 mutations do not confer 3-AT resistance in any of the strains containing the modified HIS3 promoters in contrast to the 3-AT phenotype conferred by not5. Further, it was found that $TAF_{II}130$ and $TAF_{II}90$ specifically co-immunoprecipitate with NOT5 but not with CCR4 (Komarnitsky et al 1998) and that GST-NOT2 and GST-NOT5 specifically interact with and retain TBP, SPT3 and several TAFs which are components of TFIID and SAGA. Interaction of NOT2 and NOT5 with SPT3 and the TAF_{II}s which are SAGA components is consistent with a recent report demonstrating a physical association of the SAGA components ADA2 and GCN5 with NOT2 (Benson et al 1998). On the other hand, NOT5 can co-immunoprecipitate TBP and $TAF_{II}130$, and GST-NOT5 can retain $TAF_{II}40$, which are present only in TFIID and not in the SAGA complex (Grant et al 1996). These results suggest that the NOT proteins may be capable of making multiple contacts with TFIID and SAGA components.

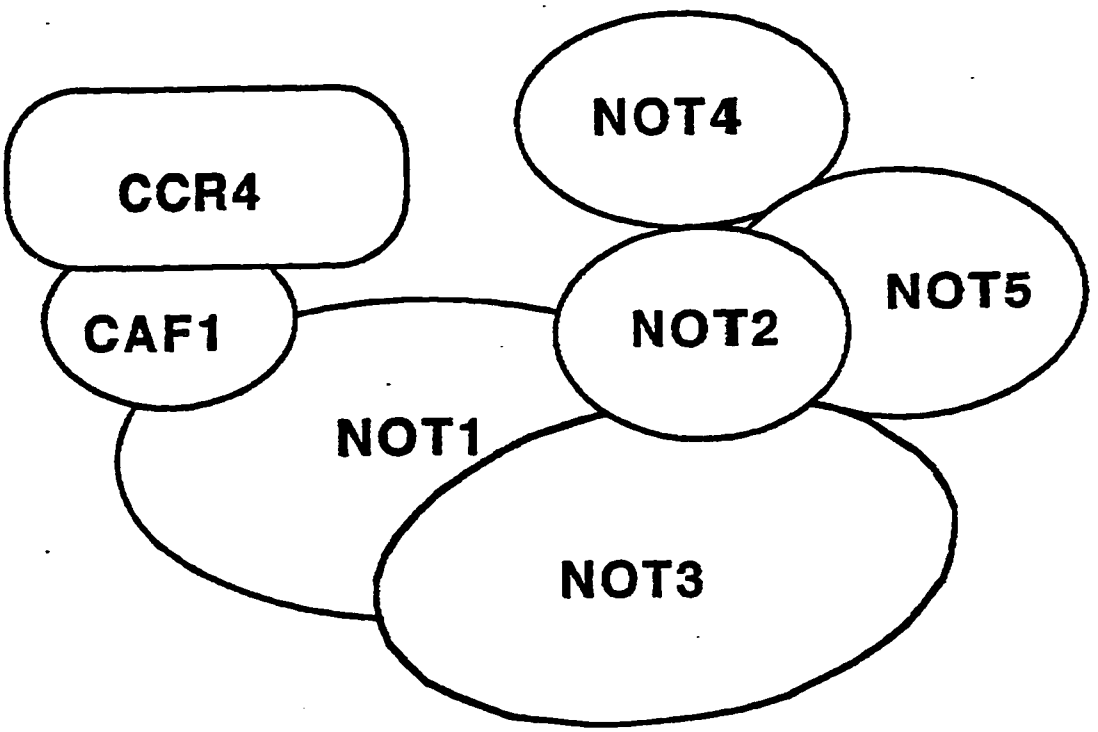


Figure 14: Proposed structure of the CCR4-NOT complex.

Taken from Bai et al 1999.

NOT2 and NOT5 interact with distinct TAF-containing complexes

The interactions between the CCR4-NOT complex and TFIID as well as SAGA were further investigated using purified TFIID and SAGA complexes. The results from these binding experiments indicate that NOT2 and NOT5 associate with components of distinct complexes. NOT5 was found to associate with and retain several proteins from purified TFIID, including $TAF_{II}130$, $TAF_{II}61$ $TAF_{II}60$, $TAF_{II}19$ and TBP. Interestingly, while NOT5 retains $TAF_{II}61$ and $TAF_{II}60$ from purified TFIID, it does not bind the same proteins when they are part of the purified SAGA complex. Similarly NOT2 retains $TAF_{II}90$ and $TAF_{II}61$ from purified SAGA but does not associate with the same proteins when they are part of the purified TFIID complex. These results demonstrate the specificity of the interactions between NOT5 and components of TFIID and between NOT2 and components of SAGA. It is also clear that the interactions of NOT5 and NOT2 with TFIID and/or SAGA in vivo may be more complex than what we observe using purified TFIID or SAGA complexes. For instance, we observe that $TAF_{II}40$ can be retained from crude extracts by GST-NOT5 but $TAF_{II}40$ as a part of purified TFIID did not bind GST-NOT5. Similarly GCN5 from crude extracts was shown to bind NOT2 (Benson et al 1998) but I found no interaction between GCN5 and NOT2 using purified SAGA. Apparently, in vivo, multiple contacts between TFIID and/or SAGA may be made to the CCR4-NOT complex, resulting in more robust interactions than what we observe in our in vitro binding experiments.

It should also be mentioned that the interactions of NOT2 and NOT5 with TFIID and SAGA components is consonant with a distinct location of NOT2 and NOT5 in the CCR4-NOT complex (Bai et al 1999). These two proteins interact with the C-terminal region of NOT1 whereas CCR4 and CAF1 bind to a central section of NOT1. In addition the NOT2 and NOT5 proteins appear to be very closely associated physically (Bai et al 1999). The general dissimilarity in function between CCR4 and CAF1 and the that of NOT2 and NOT5 can now be better understood as not only as a result of physical separation of these subsets of proteins but also from the distinctiveness in physical interactions. CCR4 and CAF1 do not interact with TAFs, TBP or GCN5 (Komarnitsky et al 1998; Chiang et al 1996) whereas NOT2 and NOT5 do. Since ccr4 or caf1 disruptions are lethal when combined with that of not2 or not5 (Bai et al 1999) it implies that CCR4 and CAF1 are acting by a separate biochemical mechanism to affect gene expression.

Based on these results two possible mechanisms can be envisaged by which the CCR4-NOT complex functions. 1) The interaction of the CCR4-NOT complex with TFIID or SAGA could disrupt these complexes thereby preventing their function. This model is based on the observation that NOT5 and NOT2 retain only a subset of proteins from TFIID and SAGA respectively.

The model that NOT5 represses TBP activity by disrupting the TFIID complex can be tested by two approaches - i) Purified TFIID can be imobilized on an anti-TBP antibody column and the affect of purified NOT5 addition on the integrity of the immobilized TFIID can be monitored by probing the column washes for

presence of TFIID components. Purified NOT2 which does not interact with TFIID can be used as a negative control. Since it is possible that the anti-TBP antibody could mask an epitope required for TFIID-NOT5 interactions, the purified TFIID complex can also be immobilized on a GST-ADR1-TADIV column (Komarnitsky et al 1998). TADIV (TransActivation Domain IV) of ADR1 has been shown to bind and retain purified TFIID in stoichiometric amounts (V. B and J.H.F. unpubl. obs.).

ii) A genetic approach to test the TFIID disruption model would utilize the K151L, K156Y allele of TBP. This allele of TBP is unable to maintain the integrity of TFIID at elevated temperatures (Ranallo et al 1999), presumably due to weaker TBP-TAF interactions. It has been shown that , at elevated temperatures several of the TAFs including $TAF_{II}130$, $TAF_{II}90$, $TAF_{II}61$ and $TAF_{II}25$ fail to associate with TBP. Therefore, if NOT5 did function by disrupting TFIID, then it is likely that overexpression of NOT5 would exacerbate the effects of the K151l, K156Y allele of TBP and perhaps result in synthetic lethality.

In case of NOT2, under conditions wherein GST-TBP was found to retain all components of SAGA tested , GST-NOT2 retained only two out of four components tested. The two components retained by NOT2 include $TAF_{II}90$ and $TAF_{II}61$. It has been reported that $TAF_{II}61$ is required for the the acetylation of nucleosomal histones by the SAGA complex (Grant et al 1998). Therefore, NOT2 could repress transcription by binding and removing $TAF_{II}61$ from the SAGA complex thereby inhibiting the nucleosomal histone acetylation (HAT) activity of the complex. This model can be tested by experiments similar to those described in (i), using

immobilized SAGA complex and purified NOT2. In addition, the effect of NOT2-TAF61 interaction on the nucleosome acetylation by SAGA can tested by carrying out nucleosomal HAT assays for SAGA in presence of purified NOT2.

2) The alternative model is that, the CCR4-NOT complex could sequester TFIID or SAGA thereby affecting TBP function at certain promoters. For example $TAF_{II}130$ and $TAF_{II}19$ have been shown to be required for transcription from non-consensus TATA elements such as the T_C element of the HIS3 gene and the TRP3 promoter (Moqtaderi et al 1996, 1998). Therefore the binding of NOT5 to $TAF_{II}130$ and $TAF_{II}19$ may modulate their activity or prevent their access to these promoters. This model can be tested by determining if purified NOT5 or CCR4-NOT complex affects the ability of purified TFIID to bind non-consensus TATA DNA sequences. In these experiments, the DNA containing a non-consenus TATA element will be immobilized on magnetic beads as described in Ranish et al 1999. Purified TFIID will be incubated with the immobilized DNA in appropriate binding buffer. Following several washes, the binding of TFIID to the DNA will be determined by Western blotting for various components of TFIID, subsequent to boiling the DNA- beads in SDS loading buffer and running it on an SDS-PAGE gel. Immobilized DNA lacking any TATA-like sequences will be used as a negative control. The binding of TFIID to non-consensus TATA elements will subsequently be carried out in the presence of purified NOT5 or purified CCR4-NOT complex, to determine if the presence of these proteins block or inhibit the ability of TFIID to bind non-consensus TATA elements.

Interaction between TFIIA and the CCR4-NOT complex

While investigating the possibility that the CCR4-NOT complex modulates the activities of factors that bind TBP, TFIIA was found to be a possible target for repression by the CCR4-NOT complex. Initial experiments indicated that increasing the concentration of TFIIA had similar affects on transcription from non-consensus TATA elements as deleting not4 or not5.Subsequent analysis indicated that this effect of TFIIA was not due to titration or squelching by individual subunits of TFIIA. It was found that the effect of overexpression of TFIIA on expression from non-consensus promoters required the function of TFIIA and interaction between TFIIA and TBP. TFIIA function as well as TBP-TFIIA interactions have been shown to be required for optimal transcription from the native HIS3 promoter and our results indicated that TFIIA-TBP interaction was also required to maintain the low -level transcription from the non-consensus TATA containing his3 promoter derivatives. This suggests that TFIIA activity as well as TBP-TFIIA interactions are required at these promoters, primarily for other functions distinct from overcoming repression by the CCR4-NOT complex. Therefore, the repressive effect of the CCR4-NOT complex on TBP function is not mediated exclusively through TFIIA. Biochemical evidence consistent with this possibility indicates that interaction between the CCR4-NOT complex and TBP is not mediated by TFIIA and does not require TBP-TFIIA interaction. However, it is possible that the NOT5-TFIIA interactions that were observed, were mediated by one or more of the TAFs that have been shown to associate with NOT5. Interaction between TAFs and TFIIA have been previously

reported (Sun et al 1994; Yokimori et al 1993). Considering the genetic evidence that TFIIA-TBP interaction is required at the native HIS3 promoter and at the his3 promoter derivatives used in this study, and the biochemical evidence that NOT5 interacts with TBP independent of TBP-TFIIA interactions, it seems unlikely that TFIIA is a direct target of the CCR4-NOT complex.

A genetic approach to test if TFIIA is a direct target of the CCR4-NOT complex would be utilize known mutations in TFIIA that are defective for a specific function such as TBP binding, DNA binding or TOA1-TOA2 interactions (Kang et al 1995; Ozer et al 1998). If one function of TFIIA is to overcome repression by the CCR4-NOT complex, then some of the effects of these TFIIA mutants should be alleviated by mutations in the NOT genes.

Another method of testing if TFIIA is a direct target is to investigate whether purified /recombinant TFIIA can associate with GST-NOT5 or GST fusions of other components of the CCR4-NOT complex. If TFIIA does associate with one or more components of the CCR4-NOT complex, then the binding domain on either subunit of TFIIA can be mapped by deletion analysis. Subsequently this CCR4-NOT binding domain on TOA1 and/or TOA2 can be mutagenized to isolate mutants that fail to associate with the CCR4-NOT complex. Since these TFIIA mutants will be unble to interact with the CCR4-NOT complex, they should exhibit phenotypes similar to that displayed by not deletions. Therefore, if TFIIA is a direct target of the CCR4-NOT complex, then TFIIA mutants that fail to interact with the CCR4-NOT complex

should allow increased expression from the T_C element as well as from the other HIS3 promoter derivatives used in this study.

If no association between purified/recombinanat TFIIA and GST fusions of CCR4-NOT components is detected, then it is likely that the NOT5-TFIIA interactions are mediated by the TAFs that have shown to associate with NOT5. This hypothesis can be tested using the K151L, K156Y allele of TBP (Ranallo et al 1999), that has been described in the previous section. Since this allele of TBP results in the disruption of TFIID at elevated temperature, it is possible that a subset of TAFs from TFIID that normally associate with NOT5, would fail to do so at elevated temperature. If under similar conditions TOA2 fails to associate with NOT5, it can be inferred that the TFIIA-NOT5 interaction is mediated by one or more components of TFIID.

The data from all the genetic and biochemical analysis described above indicate that components of TFIID and SAGA are targets for repression by the CCR4-NOT complex. Hence the effects of not4 or not5 deletion on transcription from the non-consensus TATA elements are presumably due to increased access or increased activity of components of TFIID and/or SAGA. It is conceivable that the effect of overexpression of TFIIA on the same promoters is due to increased access of TFIIA to these promoters where it could overcome the repressive effects of the NC2 complex or MOT1.

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APPENDIX A

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The NOT proteins are part of the CCR4 transcriptional complex and affect gene expression both positively and negatively

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The CCR4 transcriptional regulatory complex consisting of CCR4, CAF1, DBF2 and other unidentified factors is one of several groups of proteins that affect gene expression. Using mass spectrometry, we have identified the 195, 185 and 116 kDa species which are part of the CCR4 complex. The 195 and 185 kDa proteins were found to be NOT1 and the 116 kDa species was identical to NOT3. NOT1, 2, 3 and 4 proteins are part of a regulatory complex that negatively affects transcription. All four NOT proteins were found to co-immunoprecipitate with CCR4 and CAF1, and NOT1 co-purified with CCR4 and CAF1 through three chromatographic steps in a complex estimated to be 1.2×10^6 Da in size. Mutations in the *NOT* genes affected many of the same genes and processes that are affected by defects in the CCR4 complex components, including reduction in *ADH2* derepression, defective cell wall integrity and increased sensitivity to mono- and divalent ions. Similarly, *ccr4*, *caf1* and *dbf2* alleles negatively regulated *FUS1–lacZ* expression, as do defects in the *NOT* genes. These results indicate that the NOT proteins are physically and functionally part of the CCR4 complex which forms a unique and novel complex that affects transcription both positively and negatively.

Keywords: activation/CCR4/NOT proteins/repression/transcription

Introduction

There are a number of general regulatory complexes that are involved in transcriptional processes. For example, in addition to the yeast holoenzyme that contains the SRB proteins (Wilson *et al.*, 1996), the SPT3–ADA2–GCN5 complex (Grant *et al.*, 1997), the NOT complex (Collart and Struhl, 1994), the PAF1 holoenzyme (Wade *et al.*, 1996) and the CCR4 complex have all been identified as playing roles in affecting gene transcription. Each of these groups of proteins appears to be unique. The interaction and functional relationship of these groups of transcriptional regulatory factors, however, remain to be clearly established. In this study, we demonstrate that the NOT protein complex is part of the CCR4 transcriptional complex and that these two groups of proteins share overlapping functions.

CCR4 affects the expression of many genes and processes in yeast. It is required for the expression of *ADH2* and other non-fermentative genes (Denis, 1984; Denis and Malvar, 1990) and for unidentified genes involved in cell wall integrity (Liu *et al.*, 1997). *ccr4* mutations result in a partial cell cycle block during telophase and increase the sensitivity of yeast cells to Li^+ and Mg^{2+} (Liu *et al.*, 1997). *ccr4* is also a suppressor of *spt10* mutations (Denis, 1984), defects which result in enhanced transcription at *ADH2* (Denis and Malvar, 1990) and other loci (Natsoulis *et al.*, 1991). In addition to acting as an activator, CCR4 has been implicated in negatively affecting gene expression as well (McKenzie *et al.*, 1993; Schild, 1995). CCR4 is a component of a multi-subunit complex (Draper *et al.*, 1994). Two of the CCR4 complex components, CAF1 (POP2) (Sakai *et al.*, 1992; Draper *et al.*, 1995), and DBF2, a cell cycle-regulated protein kinase (Toyn *et al.*, 1991), function to control many of the same processes as CCR4 (Liu *et al.*, 1997). While none of these genes by themselves are essential, the phenotypes conferred by the *ccr4*, *caf1* and *dbf2* mutations indicate that the CCR4 complex is required for optimal and proper expression of many genes. The evolutionary conservation of CAF1 across eucaryotes (Draper *et al.*, 1995) further suggests that this complex plays an important role in eucaryotic gene control. Although the mechanism of how CCR4 functions remains unclear, the site of CCR4 action at the *ADH2* locus has been shown to occur at a post-chromatin remodeling step (Verdone *et al.*, 1997).

In addition to CAF1 and DBF2, the CCR4 complex contains several unidentified proteins, 195, 185, 140 and 116 kDa in size (Draper *et al.*, 1994). Our initial attempt at cloning the corresponding genes for these proteins by two-hybrid analysis was unsuccessful (Draper *et al.*, 1995; Liu *et al.*, 1997). Mass spectrometry has recently become the method of choice for rapid and unambiguous identification of gel-separated proteins. Large-scale analysis of yeast proteins is now possible (Shevchenko *et al.*, 1996), and entire yeast protein complexes can be studied (Lamond and Mann, 1997; Neubauer *et al.*, 1997). Here, we have used these methods to identify the 195, 185 and 116 kDa species of the CCR4 complex. The 185 and 195 kDa species were found to be NOT1 and the 116 kDa species was found to be NOT3.

The *NOT* genes have been identified as encoding a group of factors involved in repressing the transcription of *HIS3* from a non-canonical TATA (Collart and Struhl, 1994). This group of proteins contains NOT1/CDC39, NOT2/CDC36, NOT3 and NOT4/MOT2/SIG1, and genetic evidence indicates that they function as a complex *in vivo* (Collart and Struhl, 1993, 1994). In addition to affecting *HIS3* expression, the *not* mutations augment the

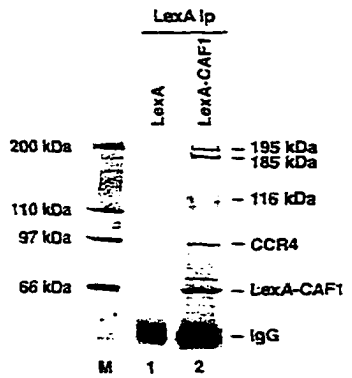

Fig. 1. Immunoprecipitation of the CCR4 complex for protein sequencing by mass spectrometry. The yeast whole cell extracts containing either LexA alone or full-length LexA–CAF1 were treated with the LexA antibody, and the resulting immunoprecipitates were subjected to SDS–PAGE. The resulting gel was stained with Coomassie blue. 'M' indicates the molecular weight standard. Lanes 1 and 2 are the immunoprecipitates from extracts containing LexA alone and LexA–CAF1, respectively. The 195, 185 and 116 kDa species in lane 2 were excised prior to mass spectrometric analysis.

expression of many genes or reporter genes, confirming their role as a repression complex (Cade and Errede, 1994; Collart and Struhl, 1994; Irie *et al.*, 1994; Collart, 1996). Of the four *NOT* genes, only *NOT1* was found to be essential. We have subsequently shown that NOT2 and NOT4 also associate with the CCR4 complex. Genetic analyses reveal that *NOT* defects result in phenotypes similar to those observed with the deletion of *CCR4* and its associated components. These results indicate that the CCR4 complex includes the NOT proteins and that this complex can affect gene transcription both positively and negatively.

Results

The 185/195 and 116 kDa proteins in the CCR4 complex are NOT1 and NOT3

To identify the proteins which associate with CCR4, the CCR4 complex was isolated by immunoprecipitation. Yeast extracts, containing either a LexA–CAF1 fusion protein or just LexA alone, were incubated with an antibody directed against the LexA protein, and the resulting immunoprecipitates were subjected to SDS–PAGE (Figure 1). After staining the proteins, the 116, 185 and 195 kDa species that specifically co-immunoprecipitated with CCR4 (Draper *et al.*, 1994) were isolated and were analyzed by mass spectrometry using the strategy previously described (Shevchenko *et al.*, 1996). A small aliquot of the peptide mixture resulting from in-gel digestion of the bands was analyzed by matrix-assisted laser desorption/ionization (MALDI). High resolution peptide mass maps were obtained of all three bands which were analyzed. Database searches with the set of measured masses resulted in the following identifications: band 116 kDa was NOT3, band 185 kDa was NOT1 and band 195 kDa was also NOT1. The identification of NOT3 was performed by MALDI peptide mapping only. The database search revealed that 26 measured peptide masses fit the sequence of NOT3 within a mass accuracy of 50 p.p.m. This corresponds to 30% of the sequence. The other two bands were subjected to both MALDI peptide mapping and mass spectrometric sequencing using nanoelectospray (Wilm *et al.*, 1996). The peptide maps covered 29% of the protein in the band migrating at 185 kDa and 32% of the protein in the band migrating at 195 kDa. The identification of the lower band is shown in Figure 2. Sequencing of 10 of the peptides derived from the 185 kDa band and eight of the peptides derived from the 195 kDa band confirmed the identification (data not shown). No peptides of the N-terminal region of the NOT1 protein were found in the analysis of the lower band. Thus, the data are consistent with the N-terminal truncation of the NOT1 protein suggested by previous studies (Collart, 1996).

NOT2 and NOT4 are also in the CCR4 complex

The NOT1 and NOT3 proteins have been shown to be part of a complex that also includes the NOT2 and NOT4 proteins (Collart and Struhl, 1994). To examine the possibility that the NOT2 and NOT4 proteins were also part of the CCR4 complex, we carried out a series of immunoprecipitation experiments. We first examined the association of NOT1 with CCR4. A LexA–NOT1 fusion was expressed in a wild-type strain. LexA–NOT1 was immunoprecipitated with the LexA antibody while the CCR4 complex was immunoprecipitated with the CCR4 antibody. The resulting immunoprecipitates were subjected to Western blot analysis (Figure 3A). CCR4 co-immunoprecipitated with LexA–NOT1 (Figure 3A, lane 3) while LexA–NOT1 along with the NOT1 proteins (185/195 kDa) were co-immunoprecipitated with CCR4 (Figure 3A, lane 5). These results confirm the protein sequencing data.

To investigate the association of NOT2 with the CCR4 complex, a LexA–NOT2 fusion was expressed in a wild-type strain, a *ccr4*Δ strain and a *caf1*Δ strain. An antibody raised against the LexA protein was used to immunoprecipitate the LexA–NOT2 fusion while antibodies raised against either CCR4 or CAF1 were used to bring down CCR4 and CAF1, respectively. The resulting immunoprecipitates were subjected to SDS–PAGE, followed by Western blot analysis (Figure 3A and B). Immunoprecipitating LexA–NOT2 with the LexA antibody resulted in co-immunoprecipitation of NOT1 from the wild-type, *ccr4*Δ and *caf1*Δ extracts (Figure 3A, lane 4, and B, lanes 1 and 2, respectively). CCR4 co-immunoprecipitated along with LexA–NOT2 and NOT1 from the wild-type strain (Figure 3A, lane 4), but not from the *caf1*Δ strain (Figure 3B, lane 1). When the CCR4 antibody was used to repeat the immunoprecipitation experiments, the NOT1 and LexA–NOT2 proteins were found to co-immunoprecipitate with CCR4 from the wild-type strain (Figure 3A, lane 6), but not from the strains lacking either CAF1 (Figure 3B, lane 3) or CCR4 (Figure 3B, lane 4). Longer exposures of the results presented in Figure 3B, lane 3, indicated that a small amount of NOT1 and LexA–NOT2 was found to co-immunoprecipitate with CCR4 from the *caf1*Δ strain (data not shown). These results indicate that NOT2 physically interacts with both CCR4 and NOT1, and that the association of CCR4 with the NOT proteins is largely dependent on the presence of

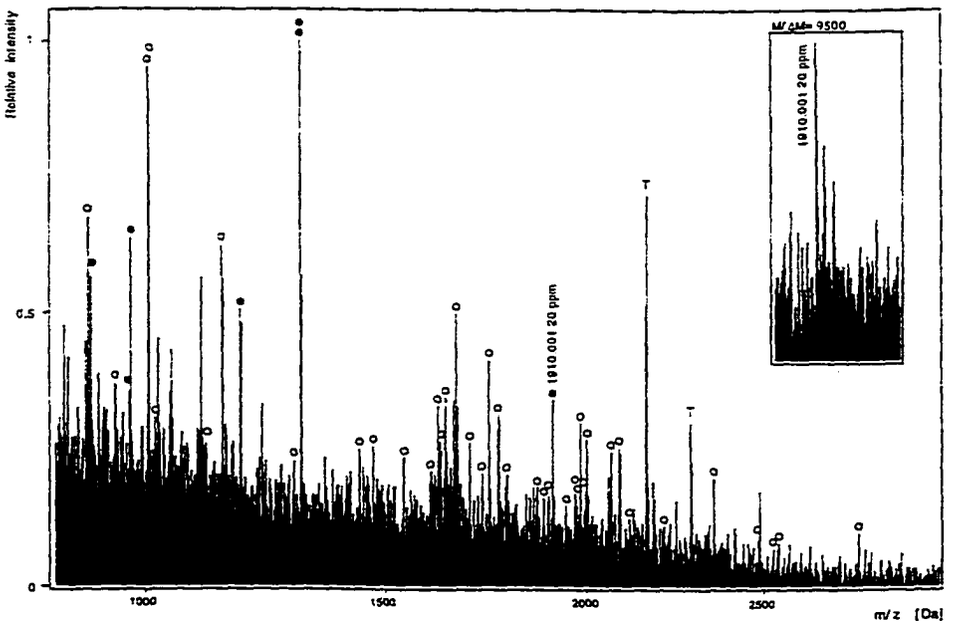

Fig. 2. Identification of the yeast protein NOT1 from the 185 kDa band by MALDI mass spectrometry. The figure shows the MALDI mass spectrum obtained after in-gel digestion of the 185 kDa band. Ion signals whose measured masses match calculated masses of tryptic peptides of NOT1 within 50 p.p.m. are indicated with circles. Filled circles mark those ion signals whose corresponding peptides were sequenced additionally by nanoelectrospray mass spectrometry. In one case, nanoelectrospray sequencing revealed two peptides for one measured peptide mass (peak at 1183.634 Da, marked by two filled circles). Ion signals corresponding to trypsin autolysis products are labeled with the letter 'T'.

CAF1. The immunoprecipitation experiments were also repeated by using the CAF1 antibody. NOT1, LexA–NOT2 and CCR4 were found to co-immunoprecipitate with CAF1 from the wild-type strain (data not shown), and NOT1 and LexA–NOT2 were co-immunoprecipitated with CAF1 from the *ccr4*Δ strain (Figure 3B, lane 6). However, NOT1 and LexA–NOT2 failed to co-immunoprecipitate with the CAF1 antibody from the *caf1*Δ strain (Figure 4B, lane 7), confirming that LexA–NOT2 does not immunoprecipitate fortuitously with the CAF1 antibody. These results also suggest that the interaction between CAF1 and the NOT proteins is CCR4 independent.

To address the question as to whether NOT4 was in the CCR4 complex, a c-Myc-tagged NOT4 fusion was expressed along with LexA–CAF1 in a wild-type strain. Extracts treated with the LexA antibody resulted in co-immunoprecipitation of c-Myc–NOT4 with LexA–CAF1, CCR4 and NOT1 (Figure 4A, lane 3), while the LexA pre-immune serum failed to immunoprecipitate these proteins (Figure 4A, lane 2). The c-Myc–NOT4 protein also co-immunoprecipitated with CCR4 and NOT1 when the extracts were immunoprecipitated with either CAF1 antibody (Figure 4A, lane 4) or CCR4 antibody (Figure 4A, lane 5). Immunoprecipitation with the c-Myc antibody, in turn, was able to bring down LexA–CAF1, CCR4 and NOT1 along with c-Myc–NOT4 (lane 6). We also immunoprecipitated the CCR4 complex from an extract prepared from a strain expressing both LexA–NOT2 and c-Myc–NOT4 fusion proteins. The resulting immunoprecipitates were analyzed by Western blot (Figure 4B). It is clear that NOT1, NOT2 and NOT4 co-immunoprecipitated with CCR4 and CAF1. Because NOT3 is also in the CCR4 complex as determined by mass spectrometry, we conclude that the complete NOT repressive regulatory complex is part of the CCR4 complex.

Two-hybrid analysis was used further to examine the interaction of the NOT proteins and the CCR4 complex components. As shown in Table I, both B42–NOT1 and B42–NOT2 interacted with LexA–CAF1, and LexA–NOT1 was found to interact with B42–CAF1. LexA–CCR4 interacted with B42–NOT1, and B42–DBF2 interacted well with LexA–NOT2. The multiplicity of these interactions confirms the above-described protein analyses.

The CCR4 complex is a unique transcriptional regulatory complex

Our previous studies on CCR4 indicated that the CCR4 complex is a transcriptional regulatory complex distinct from that of several other complexes such as the SNF/SWI complex, the yeast holoenzyme and the putative SPT4, 5, 6 complex (Denis *et al.*, 1994). The size of the CCR4 complex was estimated following Superose 6 gel filtration chromatography. As shown in Figure 5A, CCR4 migrated in two separate peaks of 1.9×10^6 and 1.0×10^6 Da. In other experiments, a small portion of CCR4 migrated at 2.0×10^5 Da, which is close to the size of CCR4 and may represent monomeric CCR4 (Figure 5C, top panel). The two larger complexes were also unaffected by prior DNase treatment, suggesting that they do not result from non-specific binding to DNA (Figure 5A, data

Fig. 3. Co-immunoprecipitation of the NOT1 and NOT2 proteins with the CCR4 complex. (A) The yeast whole cell extracts containing either LexA–NOT1 or LexA–NOT2 were treated with LexA antibody (lanes 3 and 4) or CCR4 antibody (lanes 5 and 6). The resulting immunoprecipitates along with the crude extracts (lane 1 and 2) were subjected to immunoblot analysis and probed with NOT1, CCR4 and LexA antibodies. (B) The yeast whole extracts containing LexA–NOT2 prepared from a *caf1*-deleted strain (lanes 1, 3 and 5) or a *ccr4*-deleted strain (lanes 2, 4 and 6) were treated with LexA antibody (lanes 1 and 2), CCR4 antibody (lanes 3 and 4) or CAF1 antibody (lanes 5 and 6). The resulting immunoprecipitates were subjected to immunoblot analysis and probed with NOT1, CCR4 and LexA antibodies. The bands beneath LexA–NOT2 in lanes 1 and 2 represent degradation products of LexA–NOT2 (data not shown).

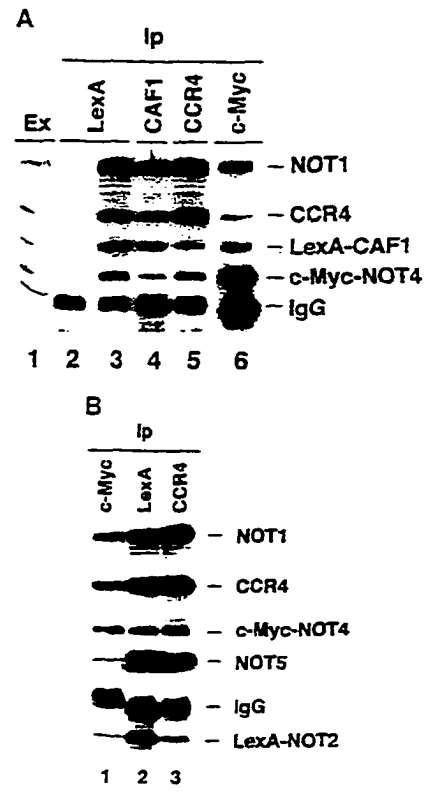

Fig. 4. Co-immunoprecipitation of the NOT1, 2 and 4 proteins with the CCR4 complex. (A) Yeast whole cell extracts containing c-Myc–NOT4 and LexA–CAF1 were treated with LexA pre-immune serum (lane 2), LexA antibody (lane 3), CAF1 antibody (lane 4), CCR4 antibody (lane 5) or c-Myc antibody (lane 6). The resulting immunoprecipitates along with the crude extract (lane 1) were subjected to immunoblot analysis and probed with NOT1, CCR4, LexA and c-Myc antibodies. (B) Yeast whole cell extracts containing LexA–NOT2 and c-Myc–NOT4 were treated with c-Myc antibody (lane 1), LexA antibody (lane 2) or CCR4 antibody (lane 3). The resulting immunoprecipitates were subjected to immunoblot analysis and probed with NOT1, CCR4, c-Myc, NOT5 and LexA antibodies.

not shown). The 1.9×10^6 Da CCR4 complex is separate from that of the SRB complex which, as analyzed on a longer Superose 6 column, migrated at 1.7×10^6 Da (Figure 5B). Moreover, in a *caf1*Δ strain, most of the CCR4 protein was found at the 1.0×10^5 Da size, indicating that the CAF1 protein is required for CCR4 association in the 1.9×10^6 and 1.0×10^6 Da complexes (Figure 5C, top two panels). A *caf1*Δ had no effect, however, on the ability of the non-CCR4 complex component, SPT10, to migrate at 1.9×10^6 Da (Figure 5C, bottom two panels),

Table I. Two-hybrid interaction assay

	β-Gal activity (U/mg)				
	B42–NOT1	B42–NOT2	B42–CAF1	B42–DBF2	B42
LexA–NOT1	–	–	130	–	3.6
LexA–NOT2	–	900	270	660	110
LexA–CCR4	330	–	1100	74	6.4
LexA–CAF1	1100	380	–	930	86
LexA	<2	<2	<2	<2	<2

LexA–CCR4, –NOT1 and –NOT2 contain full-length CCR4, NOT1 and NOT2. LexA–CAF1 contains residue 127–444 of CAF1. All LexA fusions contain residues 1–202 of LexA. B42–NOT1, –NOT2 and –DBF2 contain full-length NOT1, NOT2 and DBF2. B42–CAF1 contains residues 148–444 of CAF1. – indicates the β-galactosidase activity is no greater than the background interaction with B42 alone.

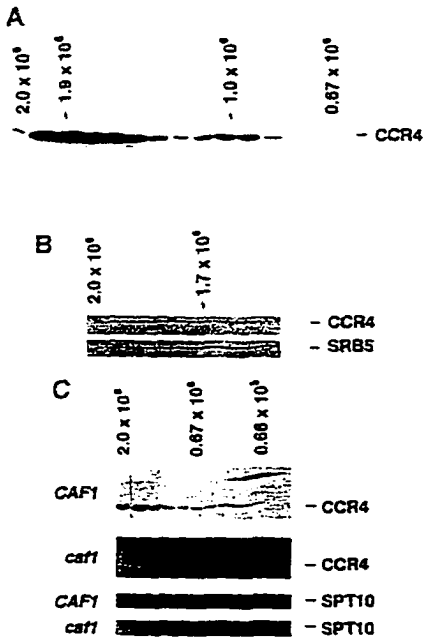

Fig. 5. Analysis of the CCR4 complexes using gel filtration chromatography. (A) Yeast whole cell extracts prepared from a wild-type strain and treated with DNase were chromatographed on a Superose 6 HR10/30 column. The resulting 1 ml fractions (30 μl of each fraction) were subjected to immunoblot analysis and probed with CCR4 antibody. The two arrows indicate the size of the two peaks containing CCR4. (B) Yeast whole cell extracts were chromatographed on an extended Superose 6 HR16/50 column. The resulting 1 ml fractions (first 12 fractions) were subjected to immunoblot analysis and probed with CCR4 and SRB5 antibodies. The arrow indicates the peak containing SRB5. (C) Yeast whole cell extracts prepared from a wild-type or a *caf1*-deleted strain were chromatographed on the Superose 6 HR10/30 column. The resulting 1 ml fractions (every other fraction is displayed) were subjected to immunoblot analysis and probed for CCR4 (top two panels) and SPT10 (bottom two panels). '*CAF1*' and '*caf1*' indicate the wild-type and *caf1*-deleted strains, respectively. The 120 kDa band that runs above CCR4 in the CCR4-probed panel represents a cell-specific protein and serves as an internal control for the experiment. Based on this control, the amount of protein loaded for the '*CAF1*' experiment was about twice that of the '*caf1*' experiment, resulting in the decreased level of CCR4 protein visible in the '*caf1*' experiment for the SPT10 Western (bottom panel).

nor on the SRB5 protein to migrate at 1.7×10^6 Da (data not shown).

To analyze the CCR4 complex further, we isolated the CCR4 complex from a strain in which the *CAF1* gene was deleted and a *CAF1* gene tagged at its C-terminus with 6×His was integrated into the genome at the *TRP1* locus. This *CAF1–6His* gene was able to complement the defect of *caf1Δ* (Liu *et al.*, 1997). The extracts prepared from this strain were first put onto a Ni^{2+}-NTA column, and the bound proteins was eluted with 250 mM imidazole. The NOT1 protein and CAF1–6His were found to co-immunoprecipitate with CCR4 when the Ni^{2+} eluate was treated with CCR4 antibody (data not shown). The Ni^{2+} eluate subsequently was loaded onto a Mono Q column, and the bound proteins were eluted in a linear salt gradient.

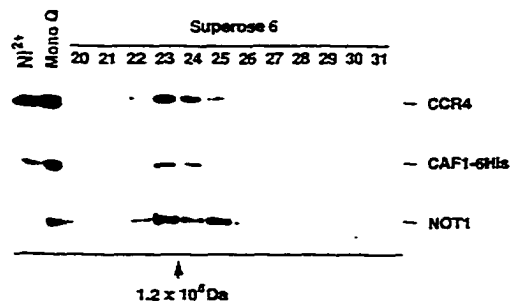

Fig. 6. Co-purification of the NOT1 protein and the CCR4 complex. Yeast whole cell extracts prepared from a *caf1*-deleted strain containing *CAF1–6His* integrated at the *TRP1* locus were chromatographed on a Ni^{2+}-NTA, Mono Q and Superose 6 HR10/30 column as described in Materials and methods. Fractions of 0.5 ml from the Superose 6 chromatography were subjected to immunoblot analysis and probed for NOT1, CCR4 and CAF1. The Mono Q lane refers to the peak fraction following Mono Q chromatography that was applied to the Superose 6 column. The Ni^{2+}-NTA eluate was not probed for NOT1. The arrow indicates the size of the peak eluted from the Superose 6 column that contains NOT1, CCR4 and CAF1–6His.

The Mono Q fractions were analyzed by Western blot using both CCR4 and CAF1 antibody, and CCR4 and CAF1–6His were found to co-elute (Liu *et al.*, 1997; Figure 6). Fractions containing both CCR4 and CAF1 were pooled and the proteins were analyzed further by Superose 6 gel filtration chromatography. The fractions from these different steps in purification were subjected to Western blot analysis. The purified CCR4 complex displayed a molecular weight of 1.2×10^6 Da following the Superose 6 gel filtration chromatography (Figure 6), corresponding closely to the 1.0×10^6 Da CCR4 complex observed in crude extracts (Figure 5A). NOT1, CCR4 and CAF1 were all found to co-purify through these three purification steps. In contrast, Western blot analysis using antibodies against SRB5 and SRB6 failed to detect either of these proteins in the Mono Q and Superose 6 fractions (data not shown). These data indicate that NOT1, CCR4 and CAF1 are components of the same complex. In addition, the 1.9×10^6 and 1.2×10^6 Da CCR4 complexes appear distinct from the yeast holoenzyme containing the SRB complex.

Mutations in the NOT genes result in similar phenotypes to those observed with ccr4 and caf1 alleles

The presence of the NOT proteins in the CCR4 complex suggest that they should function to control similar genes and processes as do CCR4 and its associated components. However, the NOT proteins have been characterized as a repression complex and CCR4 is generally considered to be an activator. To address this issue, we analyzed the effect of *not* mutations on several processes known to be affected by *ccr4*. The results from the phenotypic analyses are summarized in Table II. Mutations in the *NOT* genes except for *NOT3* reduced *ADH2* expression under non-fermentative conditions, indicating that the NOT proteins can act as activators. A *not4* allele was also capable of suppressing the enhanced *ADH2* expression that is caused

Table II. Phenotypic analysis

Strains	ADH II	*spt10* ADH II	Caffeine 8 mM	37°C YD	37°C YD 1 M sorbitol	Mg^{2+} 750 mM	Stauro 1 mg/ml	3 AT 20 mM
wt	2400	91	–	–	+	+	–	–
ccr4	400	23	–	–	+	–	–	–
caf1	1000	7	w	–	+	–	–	w/–
not1	1300	78	–	w	w	–	–	+
not2	340	86	–	–	+	–	w	+
not3	2500	N.D.	+	+	+	–	w	+
not4	1200	13	w	–	w	w	–	+

Growth was scored on YD plates as supplemented with 8 mM caffeine, 1 mg/ml of staurosporine (stauro), 750 mM $MgCl_2$ or 1 M sorbitol as indicated. 3AT: growth was scored on minimal plates lacking histidine and containing 20 mM 3-aminotriazole (3AT) using strains isogenic to KY803 (wt) containing the YCp88-Sc4363 plasmid (Collart and Struhl, 1994). Strains used for monitoring caffeine, Mg^{2+}, temperature and staurosporine sensitivity were KY803 (wt), EGY188-1 (*ccr4*), EGY188-c1 (*caf1*), MY8 (*not1*), MY16 (*not2*), MY508 (*not3*) and MY537 (*not4*). Wild-type strain EGY188 gave the same results as KY803. ADH II activities (mU/mg) represent the average of at least three determinations and were conducted following growth at 30°C on YEP medium containing 3% ethanol. No effect was observed in the *not* mutations on ADH II activity under glucose growth conditions (data not shown). The SEM for the ADH II activities was <20%. For ADH II assays, the following strains were used: wt, KY803-Δ3; *not1*, MY8-Δ1; *not2*, MY16-Δ1; *not3*, MY25-Δ1; *not4*, 612-1d-n4; and for *spt10* ADH II assays the strains were: wt, *spt10* segregants from cross 808-5c and 612-1d-n4; *not1*, *spt10 not1-2* segregants from cross MY8Δ1 and 1366-4a; *not2*, *spt10 not2* segregants from cross 808-5c and MY16-Δ1; *not4*, *spt10 not4* segregants from cross 808-5c and 612-1d-n4. The isogenic parent for 612-1d-n4 is 612-1d whose ADH II activity is 3000 mU/mg. ADH II and *spt10* ADH II activities for *ccr4* and *caf1* strains are taken from Denis (1984) and Draper *et al.* (1995). N.D., not done; '+', good growth; 'w', weak growth; '–', no or poor growth.

by an *spt10* defect. All of the *not* alleles except for *not3* also displayed sensitivity to caffeine, a phenotype resulting from defects in cell wall integrity, which is shared by the *ccr4*, *caf1* and *dbf2* alleles (Liu *et al.*, 1997). *ccr4*, *caf1* and *dbf2* mutations also result in temperature- and/or cold-sensitive phenotypes that are suppressible by 1 M sorbitol, confirming their roles in control of cell wall integrity (Liu *et al.*, 1997). In agreement with this phenotype, it has been shown previously that a *not4* allele confers a temperature-sensitive phenotype that is suppressible by 1 M sorbitol (Cade and Errede, 1994). We subsequently found that the *not2* ts phenotype was also relieved by 1 M sorbitol (Table II). Also, the caffeine-sensitive phenotype of *not4* was suppressed by 1 M sorbitol (data not shown). In agreement with these results, *not2*, *not3* and *not4* alleles were sensitive to staurosporine, an inhibitor of PKC1, indicative of cell wall defects. Moreover, *not1*, *not2* and *not4* alleles were sensitive to 0.04% SDS, another phenotype indicative of a defect in cell wall integrity (Igual *et al.*, 1996) also displayed by *ccr4*, *caf1* and *dbf2* alleles (data not shown). Furthermore, *not1*, *not2* and *not4* alleles were sensitive to high concentrations of the divalent cation, Mg^{2+}, as are *ccr4*–, *caf1*– and *dbf2*-containing strains (Table II). These results indicate that defects in the NOT factors result in phenotypes consistent with the NOT proteins functioning in processes similar to CCR4, CAF1 and DBF2.

The CCR4 complex has positive and negative effects on gene transcription

To address whether the CCR4 complex components can act as repressors in a manner similar to that observed for the NOT proteins, we examined the effect of *ccr4*, *caf1* and *dbf2* defects on *FUS1–lacZ* expression. Mutations in *NOT* genes result in increased expression of the *FUS1* gene or the *FUS1–lacZ* reporter gene in the absence of pheromone stimulation (Cade and Errede, 1994; Collart and Struhl, 1994; Irie *et al.*, 1994). As shown in Figure 7A, deletion of *CAF1* caused a 5-fold increase in β-galactosidase activity from the *FUS1–lacZ* reporter, while deletion of *CCR4* and *DBF2* resulted in an increase of β-galactosidase activity of ~2- and 3-fold, respectively. These results are similar to the 2- to 5-fold effects observed for the *not* effects on the *FUS1* promoter. The *ccr4*, *caf1* and *dbf2* effects on the *FUS1–lacZ* reporter were specific to the *FUS1* promoter since *ccr4*, *caf1* and *dbf2* had very different effects on other *lacZ* reporters carrying different promoters (see below).

To extend the comparison of the NOT proteins and the CCR4 complex components, we examined the effects of their mutations on several other reporter genes. All reporter genes, including the *FUS1–lacZ* reporter, are derived from a *UAS-less lacZ* reporter. As shown in Figure 7B, mutations in the *NOT* genes, *CCR4* and *CAF1* resulted in decreased expression of the *CYC1–lacZ* reporter gene (dependent on the HAP2, 3, 4 and 5 activator complex), in which the *ccr4*, *caf1* and *not2* alleles had the greatest effects. The effect on *CYC1–lacZ* expression was more severe when cells were supplied with a non-fermentable carbon source, such as ethanol and glycerol, than with glucose, but *ccr4*, *caf1* and *not3* also had effects under glucose growth conditions. The observed effects on the derepressed expression of the *CYC1–lacZ* reporter was not due to general effects on the plasmid or *lacZ* expression since the *FKS1–lacZ* reporter was largely unaffected under non-fermentative growth conditions by these same mutations (Figure 7C). These data confirm that, as observed with effects on *ADH2* expression, the *NOT* genes can also be involved in the activation of gene transcription.

Though the *NOT* genes and *CCR4* behave similarly, some variations in their effects on gene transcription were observed when we examined other *lacZ* reporter genes. In the case of *HO–lacZ* expression, *not1*, *not2*, *not4* and *dbf2* defects increased β-galactosidase activity while the strains containing deletion of *ccr4*, *caf1* or *not3* showed reduced β-galactosidase activity (Figure 7C). When the *FKS1–lacZ* reporter was examined, variation in the effects was again observed. The *not1*, *not4* and *dbf2* alleles caused 2-fold increases in β-galactosidase activity, while either *ccr4* or *not3* caused reductions in β-galactosidase activity

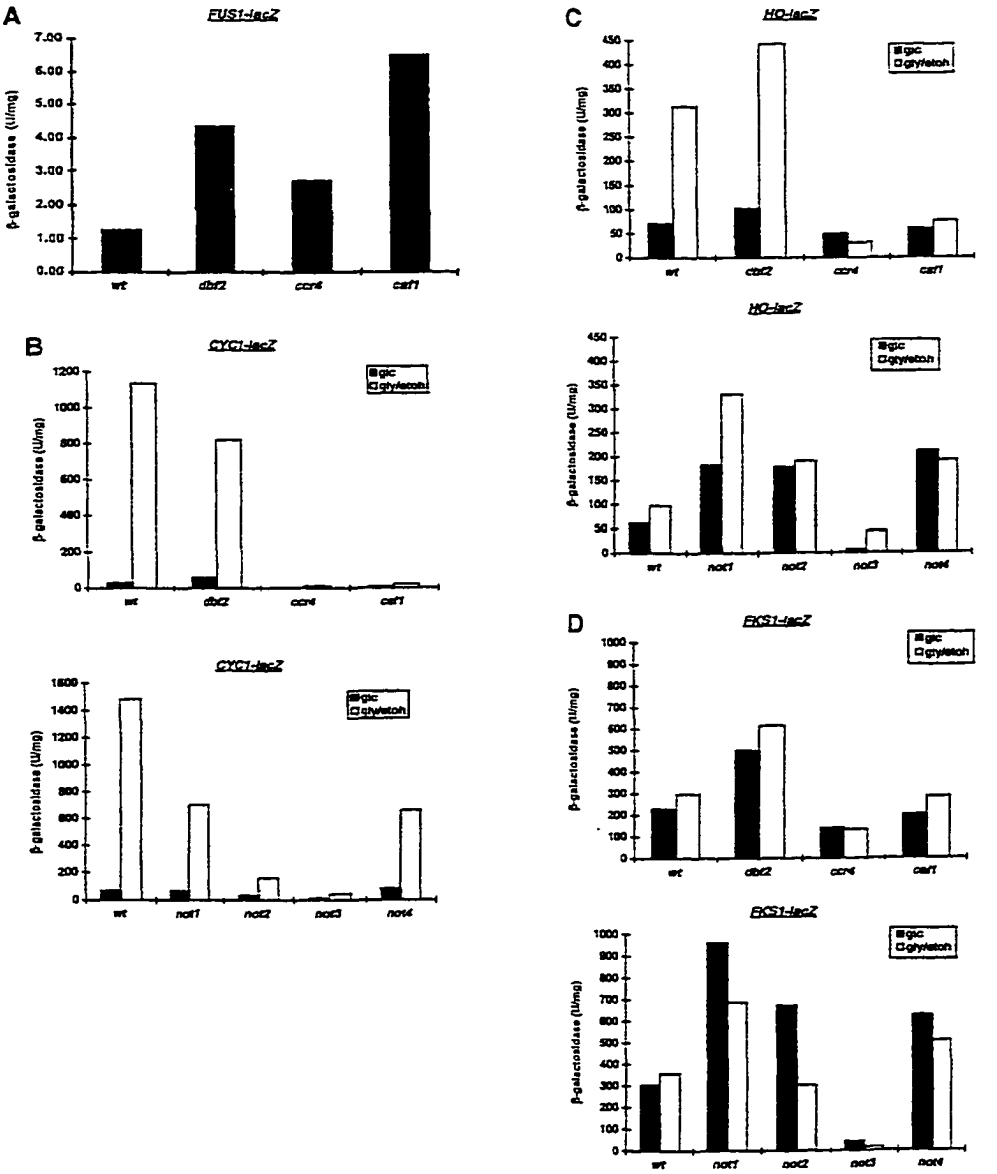

Fig. 7. Effects of the *ccr4*, *caf1*, *dbf2* and *not* mutations on regulation of gene expression. (A) β-Galactosidase activity in strains (grown on minimal medium lacking uracil and supplemented with 8% glucose) carrying a plasmid-borne *FUS1–lacZ* reporter gene without pheromone stimulation. Values are averages for at least five transformants, and the standard error of the mean (SEM) was <20% except for strain EGY188 in which it was 35%. wt, EGY188; *dbf2*, EGY188-d2; *ccr4*, EGY188-1-1; and *caf1*, EGY188-c1-1. (B) β-Galactosidase activity in strains carrying a plasmid-borne *CYC1–lacZ* reporter, LG265UP1, containing the upstream element for the HAP2, 3, 4 and 5 activator (Guarente and Mason, 1983). The upper panel displays the effects of *ccr4*, *caf1* and *dbf2* mutations on the expression of the *CYC1–lacZ* reporter while the lower panel gives the effects of the *not* mutations on expression of the same reporter. Values are averages for at least four transformants, and the SEM was <25% for glucose-grown culture whereas, for non-fermentative culture the SEM was <30%, except for strains EGY188-1-1 and EGY188-c1-1 in which it was <50%. Strains used for the upper panels for (B), (C) and (D) are the same as (A), and for the lower panels strains are: wt, KY803; *not1*, MY8; *not2*, MY16; *not3*, MY25; and *not4*, MY20. (C and D) The same experiments as (B) except that the *CYC1–lacZ* reporter was replaced by either a *HO–lacZ* reporter [containing the complete upstream sequence of the *HO* gene, plasmid BA161 (Breeden and Nasmyth, 1987)] (C) or a *FKS1–lacZ* reporter (plasmid pF712-380, Igual *et al.*, 1996) (D). Values are averages for at least three transformants, and the SEM was <25%.

(Figure 7D). The *not2*, and *caf1* alleles showed less dramatic or no effects on *FKS1–lacZ* expression. These results indicate that different components of the CCR4 complex have similar but not necessarily identical effects on gene expression.

We also analyzed the effect of *caf1* and *ccr4* defects on *HIS3* gene expression under conditions when the GCN4 activator is disabled. Using strain KY803, in which *not* mutations cause resistance to 20 mM 3-aminotriazole (3AT) whereas the parent strain is sensitive (Collart and Struhl, 1994) (Table II), we deleted *CCR4* and *CAF1*. A *ccr4* disruption in this strain background did not result in any enhanced *HIS3* expression and resistance to 20 mM 3AT whereas a *caf1* disruption resulted in weak growth at 20 mM 3AT, indicative of a slight increase in *HIS3* expression (Table II).

Discussion

The NOT negative regulatory complex is physically associated with the CCR4 transcriptional regulatory complex

Using mass spectrometry, we have identified the 195, 185 and 116 kDa species of the CCR4 complex. The 195 and 185 kDa species were found to be NOT1 and the 116 kDa species was found to be NOT3. The 185 kDa species is an apparent degradation product of NOT1 and is missing ~100 amino acids from the N-terminus. The 195 and 185 kDa species were also shown to react specifically with an antibody raised against a GST–NOT1 fusion protein. The NOT1 and NOT3 proteins are part of the NOT negative regulatory complex containing four proteins (NOT1, NOT2, NOT3 and NOT4) (Collart and Struhl, 1994). We subsequently showed by co-immunoprecipitation that the NOT2 and NOT4 proteins were also associated with the CCR4 complex. The interactions between the NOT proteins and the CCR4 complex were also confirmed by two-hybrid analysis (Table II). These results imply that there exist multiple interactions among these components and provide additional evidence that NOT proteins are part of the CCR4 complex. Recently, another component of the NOT complex, the NOT5 protein, has been found to be functionally and physically associated with the other NOT proteins (Oberholzer and Collart, 1998). We have since shown that NOT5 also specifically co-immunoprecipitates with CCR4 and CAF1 (Figure 4B; data not shown), suggesting that it too is part of the CCR4–NOT complex.

We also showed that NOT1, CCR4 and CAF1 co-purified through three different chromatographic steps using a CAF1–6His fusion to aid in the isolation of the CCR4 complex. This purified CCR4 complex containing the CAF1–6His fusion was eluted from a Superose 6 column with an estimated mol. wt of 1.2×10^6 Da. During the purification, the majority of CCR4 was found to be associated with CAF1–6His. Determination of the size of the CCR4 complex by gel filtration from a wild-type strain indicated that CCR4 and CAF1 were part of large complexes with estimated mol. wts of 1.9×10^6 and 1.0×10^6 Da. We have not been able to isolate the 1.9×10^6 Da CCR4 complex using the 6His-tagged CAF1 or CCR4, partly as the result of reduced levels of the 1.9×10^6 Da complex in these strains (unpublished observations). It is also possible that we would not be able to isolate the larger CCR4 complex using the methodology employed in this report.

These findings confirm that the CCR4 complex is truly a multi-subunit complex. The immunoprecipitation results, the co-purification of NOT1 with the CCR4 complex, the two-hybrid analysis and the previous studies on the NOT complex strongly implicate the NOT2, 3 and 4 proteins as being components of the 1.2×10^5 Da CCR4 complex, which can be considered the core CCR4–NOT complex. It remains possible, however, that other forms of the NOT complex may exist, especially since a previously identified NOT complex was found to be only 6×10^5 Da in size (Collart and Struhl, 1994).

By several criteria, the CCR4 complexes appear distinct from the yeast holoenzyme. First, neither CCR4 nor CAF1 were found to be in purified preparations of the yeast holoenzyme (Draper *et al.*, 1995). Second, SRB5 migrated in a complex that was slightly smaller than the 1.9×10^6 Da CCR4 complex (Figure 5B). Third, SRB proteins did not co-purify with the 1.2×10^6 Da complex. A number of other proteins were checked for their presence in the 1.2×10^6 Da complex or for their ability to co-immunoprecipitate with CCR4 or CAF1. RPB1, MOT1, SPT6, SPT10, ADA2, SIN3, SIN4 and several SNF/SWI proteins were all shown not to be part of the CCR4 complex (Denis *et al.*, 1994, unpublished observations). These results place the CCR4 complex, containing the NOT proteins, as a unique and novel transcriptional regulatory group of proteins.

The role of the CAF1 protein in this complex was elucidated partly through the analysis of the effects of *caf1* defects on the association of CCR4 protein with the complex. Disruption of *caf1* effectively removed CCR4 protein from the 1.9×10^6 and 1.2×10^6 Da complexes. Correspondingly, CCR4 did not immunoprecipitate well with the NOT proteins in a *caf1* background. In contrast, a *ccr4* disruption did not affect CAF1 immunoprecipitation with the NOT proteins (Figure 3B) nor did it affect CAF1 association in the 1.9×10^6 Da complex (unpublished observation). These data indicate that CCR4 association in the complex depends on the presence of CAF1. Consistent with this conclusion is the observation that high copy expression of *CCR4* can complement a *caf1* defect (Hata *et al.*, 1997); increased levels of CCR4 would be able to associate by mass action in the CCR4 complex even in the absence of CAF1. High copy expression of *CAF1* cannot complement a *ccr4* defect (Hata *et al.*, 1997), however, apparently because CCR4 plays an essential role that increased levels of CAF1 cannot duplicate.

The NOT complex is functionally associated with the CCR4 complex

The previous studies on the *NOT* genes clearly demonstrated that they played a negative regulatory role in gene transcription. Our finding that this complex physically associates with the CCR4 complex would suggest that it should also be positively involved in gene transcription. By examining the defects of the *NOT* genes on *ADH2* expression, we were able to demonstrate that mutations in the *NOT* genes, with the exception of *NOT3*, caused a reduction of *ADH2* gene expression under glucose-derepressed conditions. This result not only establishes

functional similarity between the NOT proteins and those in the CCR4 complex but also suggests that the NOT complex is involved in activation of gene transcription.

A positive role for the NOT complex in gene transcription was demonstrated further by the observation that a *not4* disruption suppressed the ability of an *spt10* mutation to cause enhanced *ADH2* expression under glucose growth conditions. The only other known alleles which confer this phenotype are *ccr4*, *caf1* and *dbf2*, all components of the CCR4 complex (Liu *et al.*, 1997). Moreover, the expression of the *CYC1–lacZ* reporter gene, containing the upstream binding site for the HAP2, 3, 4 and 5 proteins, was reduced by defects in the *NOT* genes. In this case, the defects in the *NOT* genes reduced *CYC1–lacZ* expression as did defects in *CCR4* and *CAF1*. This reduction of *CYC1–lacZ* expression by the *not* alleles occurred primarily under non-fermentative growth conditions. Like CCR4 and CAF1, the NOT proteins may play a special role in aiding the expression of non-fermentative genes.

In addition to their similar effects on non-fermentative gene expression, the CCR4 complex components and the NOT proteins shared other phenotypic similarities. Mutations in all of these genes except that of *NOT3* resulted in increased caffeine sensitivity. This phenotype appears to be the result of impaired formation of the cell wall (Levin and Bartlett-Heubusch, 1992). Moreover, the cold-sensitive phenotype of *ccr4* and the ts phenotypes of *dbf2*, *caf1*, *not2* and *not4* were all suppressed by osmotic stabilizing agents such as sorbitol, confirming a defect caused by the alleles in terms of cell wall integrity. *ccr4*, *caf1*, *dbf2*, *not2*, *not3* and *not4* alleles were also sensitive to staurosporine, indicative of a cell wall integrity problem. Increased sensitivity to mono- and divalent cations is also a phenotype associated with *ccr4*, *caf1* and *dbf2* alleles, and a similar sensitivity was observed for the *not* alleles.

Whereas the CCR4 complex previously had been ascribed a positive role in gene expression, its association with the NOT proteins implicates them in affecting gene expression in a negative way as well. Previous data have indicated that *ccr4* mutations can negatively affect gene expression in the methionine biosynthetic pathway (McKenzie *et al.*, 1993). In this case, a *ccr4* mutation acted in a manner similar to such other negative regulators as SPT21, RPD3 and RPD1 (SIN3). Also, *ccr4* and *caf1* alleles cause increased resistance to X-ray radiation, presumably by releasing negative control of genes involved in the *RAD51* and *RAD52* pathway (Schild, 1995). Furthermore, the original identification of a *caf1* mutation (*pop2*) involved its negative control of *PGK1* expression during stationary phase (Sakai *et al.*, 1992). We further showed that *CCR4*, *CAF1* and *DBF2* negatively affect *FUS1–lacZ* expression in the same manner as did the *NOT* genes (Cade and Errede, 1994; Collart and Struhl, 1994). These observations indicate that the CCR4 complex components, like the NOT proteins, can play negative roles in controlling gene expression. Therefore, the protein association of the NOT proteins with components of the CCR4 complex results in overall similar control of gene expression and other processes.

Notwithstanding the above-described similarities, the *CCR4*, *CAF1*, *DBF2* and *NOT* genes were found to differ in their effects in some cases. This was observed most obviously with the *HO–lacZ* and *FKS1–lacZ* reporter genes and with *HIS3* expression. These data suggest that although all the CCR4 complex components can share common functions, the individual components of this complex can behave differently in regulating different genes.

The fact that these various proteins can affect expression both positively and negatively suggests that the role of CCR4, CAF1 and the associated NOT proteins may be more versatile than previously indicated. The demonstration that CCR4 acts at the *ADH2* locus at a post-chromatin remodeling step (Verdone *et al.*, 1997) is consistent with the model that the NOT proteins act to regulate TATA box-binding protein (TBP) use of non-consensus TATAA sequences (Collart and Struhl, 1994; Collart, 1996). The function of the CCR4–NOT complex is also clearly affected by the sequences that lie upstream of the TATAA (Figure 7). It is likely that sequence-specific activator binding or chromatin structure influences the NOT proteins and other CCR4 complex components in their mode of action. Because of the size of the CCR4 complex and the number of its components, it is highly likely that individual factors, while showing overall functional similarity to other components in the complex, will play somewhat different roles in transcription. Individual proteins may be the targets of different regulatory factors and regulatory processes. For instance, DBF2 is a cell cycle-regulated protein kinase, and defects in *DBF2* cause a telophase block. *CAF1* and *CCR4* are themselves not cell cycle regulated, and mutations in them cause only a partial late mitotic defect (Liu *et al.*, 1997). Clearly, the CCR4 transcriptional complex does not act by itself, and identifying its contacts with the several other known protein complexes involved in transcription remains a major focus to understanding how the CCR4 complex and its individual proteins function.

Table III. Yeast strains

Strains	Relevant genotypes
808-5c	*MATα spt10::LEU2 ura3 his3 leu2 trp1*
KY803	*MATa trp1Δ1 ura3-52 gcn4 leu2::PET56*
KY803-t-1	isogenic to KY803 except *ccr4::URA3*
KY803-c1-1	isogenic to KY803 except *caf1::LEU2*
MY8	isogenic to KY803 except *not1-2* and *MATα*
MY16	isogenic to KY803 except *not2-1*
MY25	isogenic to KY803 except *not3-2*
MY508	isogenic to KY803 except *not3::URA3*
MY537	isogenic to KY803 except *not4::URA3*
KY803-Δ3	isogenic to KY803 except *adh1::URA3*
MY8-Δ1	isogenic to MY8 except *adh1::URA3*
MY16-Δ1	isogenic to MY16 except *adh1::URA3*
MY25-Δ1	isogenic to MY25 except *adh1::URA3*
612-1d-n4	*MATa adh1-11 ura3 his3 trp1 leu2 not4::URA3*
EGY188	*MATa adh1-11 ura3 his3 trp1 LexA–LEU2*
EGY188-c1	isogenic to EGY188 except *caf1::URA3*
EGY188-1	isogenic to EGY188 except *ccr4::URA3*
EGY188-d2	isogenic to EGY188 except *dbf2::LEU2*

Materials and methods

Strains and culture

Yeast strains are listed in Table III. Growth on YD solid medium was done with Petri plates containing YEP (1% yeast extract and 2%

bactopeptone) supplemented with 2% glucose and 2% bactoagar. β-Galactosidase assays were conducted as described (Cook *et al.*, 1994) on minimal medium lacking uracil that was supplemented either with 8% glucose or with 2% each of glycerol and ethanol. ADH II assays were conducted as described (Cook *et al.*, 1994).

Immunoprecipitation

Immunoprecipitations were carried out as described previously (Draper *et al.*, 1994; Liu *et al.*, 1997). To isolate the CCR4 complex for protein sequencing, yeast whole cell extracts prepared from a 400 ml overnight culture were mixed with 20 µl of affinity-purified LexA antibody for 45 min. To this was added 300 µl of a 50% protein A–agarose slur, and the incubation was continued for an additional 30 min. The resulting immunoprecipitate was resuspended in 150 µl of 2× SDS sample buffer and boiled for 5 min. The sample was divided and loaded onto three lanes. After electrophoresis, the gel was stained in Coomassie blue solution for 2 h and destained overnight. The protein bands of interest were excised and subjected to mass spectrometric analysis. Immunoblot analysis was carried out according to the described procedures (Liu *et al.*, 1997). The immunoblot results were analyzed by an Arcus II Scanner (Agfa-Gevaert, N.V., UK) and Adobe Photoshop 3.0 (Adobe Systems Inc., USA).

Protein identification by mass spectrometry

Techniques and strategy of analysis were as previously described (Shevchenko *et al.*, 1996). Gel pieces were washed, 'in-gel' reduced, S-alkylated, and protein enzymatically degraded with trypsin as described (Wilm *et al.*, 1996). After 3 h, ~2% of the digest product was applied on a micro-crystalline layer—a mixture of α-cyano-4-hydroxycinnamic acid and nitrocellulose (Jensen *et al.*, 1996)—and analyzed by MALDI-time of flight mass spectrometry. The mass spectrometer (Bruker Reflex, Bruker-Franzen, Bremen, Germany) was equipped with delayed ion extraction. For peptide sequencing by nanoelectrospray mass spectrometry, the remaining product was extracted, concentrated and desalted on a 100 nl R2 Poros microcolumn, and eluted in 2×0.5 µl of 60% methanol, 5% formic acid into a nanoelectrospray spraying needle as described (Wilm and Mann, 1996; Wilm *et al.*, 1996). Analyses were performed on a triple quadrupole mass spectrometer (API III, Perkin-Elmer Sciex, Toronto, Canada). Database searches by peptide mass maps and by peptide sequence tags (Mann and Wilm, 1994) were performed with the program PeptideSearch using a comprehensive non-redundant database currently containing >230 000 entries.

Purification of the CCR4 complex

Yeast whole cell extracts were prepared by a modification of the method of Liu *et al.* (1997) in which 3× buffer A [50 mM Tris-OAc, pH 7.9/150 mM KOAc/20% glycerol/0.2% Tween-20/2 mM β-mercaptoethanol (β-ME)/2 mM MgOAc plus protease inhibitors] was used to resuspend cell pellets (140 g of wet cells). The clear extract (120 ml) was applied to a 4 ml Ni^{2+}-NTA column. The bound proteins were eluted in 250 mM imidazole in buffer A. The resulting Ni^{2+} eluate (12 ml) was applied to a Mono Q HR5/5 column and the protein fractions were then eluted in a 20 ml linear 100–2000 mM gradient of KOAc in buffer B: 50 mM Tris-OAc, pH 7.9/100 mM KOAc/20% glycerol/0.02% Tween-20/1 mM β-ME/2 mM MgOAc/1 mM EDTA, plus protease inhibitors including 2 µl/ml of leupeptin (2 mg/ml), pepstatin A (1 mg/ml), chymostatin (5 mg/ml) and benzamidine (500 mM), and 10 µl/ml of 500 mM phenylmethylsulfonyl fluoride (PMSF). The fractions containing CCR4 and CAF1–6His were pooled (2 ml in total) and subjected to ultrafiltration using a Centricon 10 device (Amicon, MA). The concentrated protein sample (200 µl) was applied to a Superose 6 HR10/30 column equilibrated in buffer G (50 mM Tris-OAc, pH 7.9/150 mM KOAc/10% glycerol/0.02% Tween-20/1 mM dithiothreitol/1 mM EDTA/2 mM MgOAc) plus protease inhibitors. The protein fractions were collected as 0.5 ml/fraction and stored at −80°C while part of the materials (10 µl for Ni^{2+}-NTA and Mono Q eluate, and 25 µl for Superose 6 fractions) was subjected to immunoblot analysis using CCR4, CAF1 and NOT1 antibodies.

Gel filtration chromatography

The Superose 6 columns HR10/30 and HR16/50 were packed with Superose 6 media, prep grade, according to the manufacturer's instructions (Pharmacia). A molecular weight standard mixture was used to calibrate the Superose 6 columns. The calibration for the HR10/30 column in buffer G plus protease inhibitors was: exclusion volumn (blue dextran, 2000 kDa) at 10 ml; thyroglobulin (669 kDa) at 15 ml; bovine serum albumin (BSA) (66 kDa) at 17.5 ml. For the HR16/50 column, the calibration was: blue dextran at 38.6 ml; thyroglobulin at 59 ml; amylase (200 kDa) at 65 ml; BSA at 69 ml; carbonic anhydrase (29 kDa) at 72.7 ml.

To analyze the CCR4 complex using gel filtration chromatography, yeast whole cell extracts prepared from a 500 ml overnight glucose-grown culture in 3× buffer A were first clarified by ultracentrifugation in a SW65 rotor at 45 000 r.p.m. for 60 min. Then 200 µl of the clear extracts were loaded onto a Superose 6 column. The resulting 1 ml fractions were subjected to Western blot analysis. For the DNase treatment experiments, yeast whole extracts were prepared in EDTA-free 3× buffer A. After ultracentrifugation, 7.5 µl of DNase I (61.5 µg/ml) were added to 1 ml of the clear extracts and the mixture was incubated at room temperature for 7 min. Then 200 µl of the mixture was analyzed on the Superose 6 column and 30 µl of the 1 ml fractions were subjected to immunoblot analysis.

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